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PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

FILING UNDER 37 C.F.R. 1.53(b)

Box Patent Application
Assistant Commissioner for Patents
Washington, DC 20231

Express Mail Label No. EL515352501US
Date of Deposit April 18, 2000

Sir:

This is a request for filing an application under 37 C.F.R. 1.53(b) of

Applicant(s): Arne Holm and Bjarne Due Larsen

Title: IMPROVED SOLID-PHASE PEPTIDE SYNTHESIS AND AGENT FOR
USE IN SUCH SYNTHESIS

55 pages of specification 12 sheets of drawings

4 sheets of ☒ executed ☐ unexecuted Declaration and Power of Attorney

☒ The filing fee is calculated as follows:

Basic Fee:	\$690.00
Total Claims: 25 - 20 = 5 x 18 =	\$90.00
Independent Claims: 6 - 3 = 3 x 78 =	\$234.00

☒ Less 50% (small entity statement enclosed) - \$507.00

Total Fee: \$507.00

A check for the required fee, estimated to be \$507.00 is herewith enclosed.

☒ Priority of Danish application 0971/96 filed on September 9, 1996 is claimed under 35 U.S.C. 119. A certified copy ☐ will subsequently be filed, ☒ has been submitted or ☐ is attached herewith.

☒ This application additionally claims priority under 35 U.S.C. §120 to and is a ☐ divisional ☐ continuation ☒ continuation-in-part of U.S. application serial no. 09/254,523, filed March 9, 1999 and also claims priority under 35 U.S.C. §120 to and is a ☐ divisional ☒ continuation ☐ continuation-in-part of PCT application no. PCT/DK97/00375, filed September 9, 1997.



09/254,523, 04/18/00

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
EXPRESS MAIL CERTIFICATE**

Assistant Commissioner for Patents
Box Patent Application
Washington, D.C. 20231

Re: "IMPROVED SOLID-PHASE PEPTIDE SYNTHESIS AND AGENT FOR
USE IN SUCH SYNTHESIS"

Applicants: Arne Holm and Bjarne Due Larsen

Sir:

Express Mail Label No.EL515352501US
Date of Deposit: April 18, 2000

I hereby certify that the following attached paper(s) or fee

1. Filing Under 37 C.F.R. 1.53(b) + fee
2. Application
3. Assignment + Fee
4. Small Entity Status Form
5. Sequence Listing + Diskette
6. Statement to support filing and submission in accordance with 37 C.F.R.
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are being deposited with the United States Postal Service "Express Mail Post Office to
Addressee under 37 C.F.R. 1.10 on the date indicated above and is addressed to the Assistant
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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Holm and Larsen

Serial No.: To Be Decided Group Art Unit: To be Decided

Filed: Concurrently Herewith Examiner: To Be Decided

For: **IMPROVED SOLID-PHASE PEPTIDE SYNTHESIS AND AGENT FOR USE IN SUCH SYNTHESIS**

VERIFIED STATEMENT CLAIMING SMALL ENTITY STATUS
(37 CFR 1.9(f) and 1.27(c))-Small Business Concern

Assistant Commissioner for Patents
Washington, DC 20231

Sir:

I hereby declare that I am

- ☐ the owner of the small business concern identified below:
☒ an official of the small business concern empowered to act on behalf of the concern identified below:

Name of ConcernZealand Pharmaceuticals A/S.....
Address of ConcernSmedeland 26B.....
.....DK-2600 Glostrup.Denmark.....

I hereby declare that the above-identified small business concern qualifies as a small business concern as defined in 13 CFR 121.3-18, and reproduced in 37 CFR 2.9(d), for purposes of paying reduced fees under section 41(a) and (b) of Title 35, United States Code, in that the number of employees of the concern, including those of its affiliates, does not exceed 500 persons. For purposes of this statement, (1) the number of employees of the business concern is the average over the previous fiscal year of the concern of the persons employed on a full-time, part-time or temporary basis during each of the pay periods of the fiscal year, and (2) concerns are affiliates of each other when either directly or indirectly, one concern controls or has the power to control the other, or a third party or parties controls or has the power to control both.

06-07

by inventor(s).....Arne Holm..and Bjarne Due Larsen.....
described in

[] application serial no., Filed

[] patent no., issued

If the rights held by the above-identified small business concern are not exclusive, each individual, concern or organization having rights to the invention is listed below* and no rights to the invention are held by any person, other than the inventor, who could not qualify as a small business concern under 37 CFR 1.9(d) or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e).

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I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b))

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

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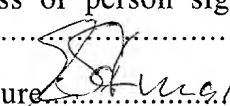
CHERYL H. AGRIS, PH.D.
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Name of person signing.....Eva Steiness.....

Title of person other than owner...PRESIDENT AND CEO.....

Address of person signing..Smedeland 26B.....

.....DK-2600 Glostrup.Denmark.....

Signature..........

Date.....11/4 - 2000.....

* NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities.
(37 CFR 1.27)

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IMPROVED SOLID-PHASE PEPTIDE SYNTHESIS AND AGENT FOR USE IN SUCH SYNTHESIS

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This application is a continuation-in-part of U.S. patent application serial no. 09/254,523, which is a continuation of PCT/DK97/00375, the contents of which are herein incorporated by reference.

10 FIELD OF THE INVENTION

The present invention relates to an improved process for the production of peptides by solid-phase synthesis. The invention also relates to agents, which are useful in solid-phase peptide synthesis.

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BACKGROUND OF THE INVENTION

Solid-phase peptide synthesis (SPPS) is a highly successful method introduced by Merrifield in 1963 (Merrifield, R.B. (1963) J. Amer. Chem. Soc. 85, 2149 - 2154).

20 Numerous peptides have been synthesized with this technique since then. Methods used in the prior art to chemically synthesize peptides and proteins are reviewed in Kent, S.B.H. (1988), Ann. Rev. Biochem. 57, 957 - 989.

Two strategies for the assembly of peptide chains by solid-phase synthesis have been
25 used, viz. the stepwise solid-phase synthesis, and solid-phase fragment condensation. In stepwise SPPS, the C-terminal amino acid in the form of an N- α -protected, if necessary, side-chain, protected reactive derivative is covalently coupled either directly or by means of a suitable linker to a "solid"- support, e.g., a polymeric resin, which is swollen in an

organic solvent. The N- α -protective group is removed, and the subsequent protected amino acids are added in a stepwise fashion. When the desired peptide chain length has been obtained, the side-chain protective groups are removed, and the peptide is cleaved from the resin. This may be done in separate steps or at the same time. In solid-phase fragment condensation, the target sequence is assembled by consecutive condensation of fragments on a solid support using protected fragments prepared by stepwise SPPS.

Over the years, two coupling strategies have been developed based on the use of different N- α -protective groups and matching side-chain protective groups. Merrifield used

tert.butyloxycarbonyl (Boc) as the N- α protective group, while 9-fluorenylmethyloxycarbonyl (Fmoc) was introduced by Carpino and Han (Carpino, L.A. and Hari, G.Y. (1972), J. Org.Chem. 37, 3404-3409). The operations involved in one cycle of chain extension in stepwise SPPC using Boc- and Fmoc -chemistries are illustrated in Figure 1 (taken from Kent, S.B.H. (1988), Ann. Rev. Biochem. 57, 957 - 989). The side-chain protection in both cases was tert.butyl, trityl and arylsulfonyl based, and these side chains were deprotected with TFA.

The N- α -Boc-protected peptide coupled to a PAM-resin was N- α -deprotected with trifluoroacetic acid (TFA). The resulting amine salt was washed and neutralized with a tertiary amine. The subsequent peptide bond was formed by reaction with an activated Boc-amino acid, e.g., a symmetric anhydride. Generally, the side-chain protection is benzyl-based, and the deprotection is made with HF or a sulphonic acid.

The N- α -Fmoc protected peptide coupled to a resin was N- α -deprotected by treatment with a secondary amine, normally piperidine, in an organic solvent, e.g., N,N-dimethyl formamide (DMF) or dichloromethane (DCM). After washing, the neutral peptide resin was reacted with an activated Fmoc-amino acid, e.g., a hydroxybenzotriazole active ester.

While the Boc- and Fmoc-strategies have been used for essentially all current practical peptide synthesis, other N- α protective groups have been proposed (Stewart, J.M. and Young, J.D., Solid phase peptide synthesis, Pierce Chemical Company (1984)). Boc forms an acid-labile urethane group, and other proposals of this category have been biphenylisopropylloxycarbonyl (Bpoc), 3,5-dimethoxyphenylisopropylloxycarbonyl (Ddz), phenylisopropylloxycarbonyl (Poc) and 2,3,5-tetramethylbenzylloxycarbonyl (Tmz). Other types of N- α protecting groups available include nitrophenylsulfonyl (Nps) which can be removed by either very dilute anhydrous acid, e.g. HCl, or by nucleophilic attack, e.g. with methyl-3-nitro-4-mercapto benzoate. Also dithiasuccinyl (Dts), which is removable by nucleophilic attack, might be used.

SPPS has the general advantage that it lends itself to fully automated or semi-automated chain assembly chemistry. A system for SPPS under low pressure continuous flow conditions was developed by Dryland & Sheppard (1986) *J. Chem. Soc. Perkin Trans. I*, 125 - 137 and was further refined (Cameron, L., Meldal, M. and Sheppard, R.C (1987), *J. Chem. Soc. Chem. Commun.* 270-272 and Meldal, M., Bisgaard Holm, C., Boejesen, G., Havsteen Jakobsen, M. and Holm, A. (1993), *Int. J. Peptide and Protein Res.* 41, 250-260 and WO 90/02605). While SPPS has now developed to be a cornerstone in protein and peptide synthesis, certain problems still remain to be solved. Since some of these problems might well be related to the peptide structure, a brief discussion regarding protein conformation is deemed proper.

Empirical predictions of protein conformations have been made by Chou & Fasman (Chou, P.Y. and Fasman, G.D. (1978), *Ann. Rev. Biochem.* 47, 251 - 276.). It is well-known that protein architectures may be described in terms of primary, secondary, tertiary and quaternary structure. The primary structure refers to the amino acid sequence of the protein. The secondary structure is the local spatial organization of the polymer

backbone without consideration of the side-chain conformation. As examples of secondary structures, α -helixes, β -sheets and β -turns, which are chain reversal regions consisting of tetrapeptides can be mentioned. The tertiary structure is the arrangement of all the atoms in space, including disulphide bridges and side-chain positions, so that all short and long-range interactions are considered. The term quaternary structure may be used to denote the interaction between subunits of the protein, e.g. the α and β -chains of hemoglobins.

Following a discussion of earlier attempts to correlate protein secondary structure with amino acid compositions, where e.g., Ser, Thr, Val, Ile and Cys were classified as "helix breakers" and Ala, Leu and Glu as "helix formers", while hydrophobic residues were classified as strong " β -formers", and proline together with charged amino acid residues as " β -breakers", Chou & Fasman made a statistical analysis of 29 proteins with known X-ray structure in order to establish prediction rules for α - and β -regions (Chou and Fasman, 1978, Ann. Rev. Biochem. 47:251-276). Based on these studies, they determined so-called propensity factors $P\alpha$, $P\beta$ and P_t which are conformational parameters expressing the positional preferences as α -helix, β -sheet and β -turn, respectively, for the natural L-amino acids forming part of proteins. For the sake of convenience, the $P\alpha$ and $P\beta$ values are listed below in Table 1. Generally speaking, values below 1.00 indicate that the amino acid in question must be regarded as unfavourable for the particular secondary structure. As an example, the hydrophobic acids (e.g. Val, Ile, Leu) are strong β -sheet formers, while the charged amino acids (e.g. Glu, Asp, His) are β -sheet breakers.

TABLE I: PROPENSITY VALUES

	$P\alpha$		$P\beta$
Glu	1.51	Val	1.70
Met	1.45	Ile	1.60
Ala	1.42	Tyr	1.47
Leu	1.21	Phe	1.38
Lys	1.16	Trp	1.37
Phe	1.13	Leu	1.30
Gln	1.11	Cyr	1.19
Trp	1.08	Thr	1.19
Ile	1.08	Gln	1.10
Val	1.06	Met	1.05
Asp	1.01	Arg	0.93
His	1.00	Asn	0.89
Arg	0.98	His	0.87
Thr	0.83	Ala	0.83
Ser	0.77	Ser	0.75
Cys	0.70	Gly	0.75
Tyr	0.69	Lys	0.74
Asn	0.67	Pro	0.55
Pro	0.57	Asp	0.54
Gly	0.57	Glu	0.37

In the α -helix structure, the spiral configuration of the peptide has been found to be held rigidly in place by hydrogen bonds between the hydrogen atom attached to the nitrogen atom in one repeating unit and the oxygen atom is attached to a carbon atom three units along the chain. If a polypeptide is brought into solution, the α -helix can be made to unwind to form a random coil, by adjustment of the pH. The transition from α -helix random coil occurs within a narrow pH. Since the hydrogen bonds are all equivalent in bond strength in the α -helix, they tend to let go all at once. The change can also be induced by heat.

The β -sheet structure consists of fully extended peptide chains in which hydrogen bonds link the hydrogen atoms on one chain to the oxygen atoms in the adjoining chain. Thus hydrogen bonds do not contribute to the internal organization of the chain as they do in the α -helix, but only bond chain to chain. Adjacent chains may be parallel or antiparallel.

β -turns are frequently observed in these parts of a peptide chain which connect antiparallel chains in a β -sheet structure. In a β -turn, the CO- and NH-groups from amino acid No. n in the peptide chain form hydrogen bond to the corresponding groups in amino acid No. $n+4$.

α -helix and β -sheet constitute strongly varying parts of the peptide conformation of proteins (from 0 to 80 %), and the remaining parts of the proteins are folded in other structures. In most proteins, sections of the peptide chains appear as irregularly folded "random coils".

Turning now to the general problems still prevailing in connection with SPPS, S.B.H. Kent (Kent, S.B.H. (1988), Ann. Rev. Biochem. 57, 957-989) highlights the synthesis of "difficult sequences". Obviously, the whole rationale of SPPS is based on a complete

N- α -deprotection prior to each of the coupling steps involved. By the same token, ideally all of the N- α -deprotected amino groups should be coupled to the reactive amino acid derivative according to the desired sequence, i.e. a complete aminoacylation should take place. Kent states that the most serious potential problem in stepwise SPPS is incomplete peptide bond formation giving rise to peptides with one or more amino acids missing (deletions), but with properties similar to the target sequence. Such incomplete couplings are more prevalent in some sequences than in others, hence the term "difficult sequences", and are apparently also more predominant in Fmoc-chemistry than in Boc-chemistry.

A number of recognized "difficult sequences" have been previously studied. During SPPS of homo-oligopeptides containing leucine or alanine using the Fmoc-strategy, ineffective N- α -deprotection with piperidine in a sequence dependent manner (B. D. Larsen, C. Larsen, and A. Holm in *Peptides 1990*, E. Giralt and D. Andreu, (Eds). 1991 ESCOM Science Publishers B.V., p. 183-185; and Larsen, B.D., and Holm, A. (1994), *Int. J. Peptide & Protein Res.* 43,1-9.) was observed. Investigations showed that this phenomenon was associated with subsequent slow or incomplete amino acid coupling and evidence for β -sheet aggregation of the growing peptide chain was presented as a cause for the difficult couplings and incomplete Fmoc-deprotections. This evidence was based on general physical-chemical observations (Larsen, B.D., and Holm, A. (1994), *Int. J. Peptide & Protein Res.* 43 , 1 - 9) and on a detailed Raman Near Infrared Spectroscopic study (Due Larsen, B., Christensen, D - H., Holm, A., Zillmer, R., and Faurskov, O. (1993), *J. Amer. Chem. Soc.* 115, 6247-6253).

Kent (Kent, S.B.H. (1988), *Ann Rev. Biochem.* 57, 957-989) proposes a number of solutions to the problem related to sequence-dependent coupling difficulties, viz. the use of heat in the coupling step and a quantitative conversion of residual unreacted resin-bound peptide chains to terminated species in a "capping" procedure. However, at

the present time, no procedure has been formulated for synthesizing these "difficult sequences" in high yield and purity.

OBJECTS OF THE INVENTION

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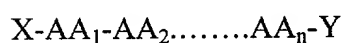
It is therefore one object of the present invention to provide an improved SPPS according to which peptides which are recognized as or prove to be "difficult sequences", can be synthesized in high yield and purity. A further object of the invention is to provide an improved SPPS which provides for reduced coupling times, not only for difficult sequences, but also for otherwise uncomplicated sequences where it is desirable to reduce the normally long coupling times. A still further object of the invention is to provide an agent or a kit for use in SPPS, whereby the above-mentioned process improvements are obtained.

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SUMMARY OF THE INVENTION

The invention is directed to a process for the production of a peptide having the structure



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wherein AA is an L- or D-amino acid residue,

X is hydrogen or an amino protective group

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Y is OH, NH₂ or an amino acid sequence comprising from 3 to 9 amino acid residues and n is an integer greater than 2 by solid phase synthesis comprising:

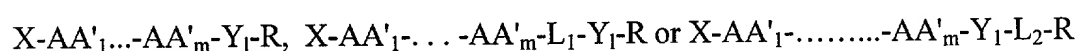
(a) coupling the C-terminal amino acid in the form of an N-alpha-protected reactive derivative to the support optionally by means of a linker, wherein said C-terminal amino acid comprises a presequence comprising from 3 to 9 amino acids and preferably from 5-7 amino acids independently selected from native L-amino acids having a side chain functionality which is protected during the coupling steps and having a propensity factor $P\alpha > 0.57$ and a propensity factor $P\beta \leq 1.10$ or the corresponding D-amino acids;

(b) adding subsequent amino acids forming the peptide sequence by stepwise coupling or coupling as a peptide fragment in the form of protected fragments;

(c) removing the protecting groups from the peptide sequence of (b) and

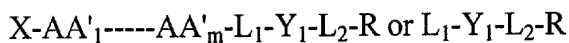
(d) optionally cleaving the presequence from the formed peptide.

Furthermore, the invention is directed to agents that are useful in solid phase peptide synthesis. These include agents having the formulae



wherein R is a solid support applicable in solid phase peptide synthesis, Y_1 is an amino acid sequence comprising from 3 to 9, preferably from 5 to 7 amino acid residues independently selected from L-amino acids having a side chain functionality which is protected during the coupling steps and having a propensity factor $P\alpha > 0.57$ and a propensity factor $P\beta \leq 1.10$, or the corresponding D-amino acid, AA' is an L or D-amino acid residue, L_1 is a linker wherein the $L_1-AA'_m$ bond is selectively cleaved, L_2 is a linker, wherein the L_2-R bond is selectively cleaved, m is zero or an integer from 1 to 40 and X is hydrogen or an amino protective group.

Alternatively, the agent may have the have the general formula



5

wherein R is a solid support applicable in solid phase peptide synthesis, Y_1 is an amino acid sequence comprising from 3 to 9, preferably from 5 to 7 amino acid residues independently selected from L-amino acids having a side chain functionality which is protected during the coupling steps and having a propensity factor $P\alpha > 0.57$ and a

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propensity factor $P\beta \leq 1.10$, or the corresponding D-amino acid, AA' is an L or D-amino acid residue, m is zero or an integer from 1 to 40 and X is hydrogen or an amino protective group, L_1 is a linker wherein the $L_1-AA'_m$ and L_2 is a linker with orthogonal cleavage conditions relative to the first linker such that the L_2-R bond is selectively cleaved.

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BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the traditional synthetic scheme followed with Boc and Fmoc-protected amino acids.

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Figure 2 is an HPLC of crude H-Ala₁₀-Lys-OH (SEQ ID NO:3) showing a substantial amount of deletion peptides (peak 1, 2, 3 and 4) and incompletely Fmoc-deprotected peptides (peak 6, 7, 8 and 9) besides the target peptide (peak 5).

25

Figure 3 is a diagram showing sequential coupling times for each of the alanines in the synthesis of H-Ala₁₀-Lys-OH (SEQ ID NO:3).

Figure 4 is an HPLC of H-Ala₁₀-Lys₃-OH (SEQ ID NO:4) showing the target peptide. No deletion peptides were observed.

Figure 5 is an HPLC of H-Ala₂₀-Lys₃-OH (SEQ ID NO:5) showing deletion peptides (peak 1, 2, 3, 4 and 5) besides the target peptide (peak 6).

- 5 Figure 6 is an HPLC of H-Ala₁₀-Lys₆-OH (SEQ ID NO:6). No deletion peptides were observed.

Figure 7 is an HPLC of H-Ala₂₀-Lys₆-OH (SEQ ID NO:7). No deletion peptides were observed.

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Figure 8 is an HPLC of H-Ala₁₀-Lys-OH (SEQ ID NO:3) prepared by introduction of an HMPA linker. Deletion peptides were observed (from Ala₁₀-Lys(tBoc)-HMPA-(Lys(tBoc))₆-resin) (SEQ ID NO:10).

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Figure 9 is a similar HPLC of H-Ala₁₀-Lys-OH (SEQ ID NO:3) prepared by using an MMA-linker showing a significant reduction the amount of deletion peptides compared to Figure 8 (from Ala₁₀-Lys-MMA-Lys(tBoc))₆-resin) (SEQ ID NO:11).

Figure 10 shows the synthetic scheme used in a specific embodiment of the invention.

20

Figure 11 is an HPLC of H-VQAAIDYING-OH (SEQ ID NO:8), Acyl Carrier Protein (ACP) (65-74). The target peptide (peak 3) was accompanied with deletion peptides (peak 2 is the des-Val peptide).

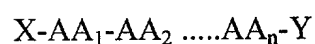
25

Figure 12 is an HPLC of H-VQAAIDYING-K₆-OH (SEQ ID NO:9), Acyl Carrier Protein (ACP) 65-74 coupled to the Lys(Boc)₆ (SEQ ID NO:22) presequence with only a minor amount of desVal peptide (peak 1).

DETAILED DESCRIPTION OF THE INVENTION

In contrast to prior art methods, the present invention is based on the incorporation of a particular presequence in the C-terminal portion of a peptide sequence attached to the solid support. This is a fundamental breach with the prior art attempts to deal with difficult sequences, where the focus was on the reaction conditions and the nature of the solid support. As further discussed below, the C-terminal sequence by which the desired peptide is attached to the solid support might also include suitable linkers in order to provide for e.g., better attachment or cleavage conditions.

Thus in a first aspect, the present invention relates to a process for the production of the peptide having the structure



wherein AA is an L- or D-amino acid residue,

X is hydrogen or an amino protective group

Y is OH, NH₂ or an amino acid sequence comprising from 3 to 9 amino acid residues and n is an integer greater than 2 by solid phase synthesis comprising:

(a) coupling the C-terminal amino acid in the form of an N-alpha-protected reactive derivative to the support optionally by means of a linker, wherein said C-terminal amino acid comprises a presequence comprising from 3 to 9 amino acids independently selected from native L-amino acids having a side chain functionality which is protected during the coupling steps and having a propensity factor $P\alpha >$

0.57 and a propensity factor $P\beta$
 ≤ 1.10 or the corresponding D-amino acids;

(b) adding subsequent amino acids forming the peptide sequence by stepwise coupling
5 or coupling as a peptide fragment in the form of protected fragments;

(c) removing the protecting groups from the peptide sequence of (b);

(d) optionally cleaving the presequence from the formed peptide.

10 It will be understood that when X is OH, the product formed after cleavage will be a peptide AA_1-AA_n-OH , i.e. $Y = OH$, while in case of X being NH_2 a peptide amide $AA_1-AA_n-NH_2$ is formed, i.e. $Y = NH_2$. In a preferred embodiment, there are from 5-7 amino acids in the presequence. In a preferred embodiment, there are from 5-7 amino
15 acids in the presequence.

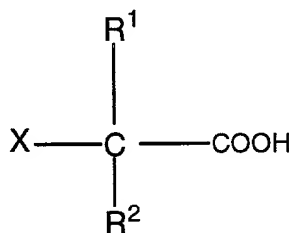
L-amino acids meeting the above-mentioned limits for the propensity factors $P\alpha$ and $P\beta$ include but are not limited to Lys, Glu, Asp, Ser, His, Asn, Arg, Met and Gln. These amino acids all have a side chain functionality selected from a carboxy, carboxamido,
20 amino, hydroxy, guanidino, sulphide or imidazole group. Presently preferred amino acids in the presequence are Lys and Glu and combinations thereof, e.g. $(Glu)_q(Lys)_p$, where $p + q$ is 3 to 9, preferably 6 to 9, and the order of Lys and Glu is arbitrarily chosen.

25 The N- α amino group of the amino acids or peptide fragments used in each coupling step should be suitably protected during the coupling. The protective group may be Fmoc or Boc or any other suitable protective group, e.g., those described above with reference to Stewart, J.M. and Young, J.D., Solid phase peptide synthesis, Pierce Chemical Company

(1984); and Peptides: Synthesis, structures and applications. Gutte, B., Ed. Academic Press Inc. 1995. It is important that the side chain functionality in the presequence is suitably protected during the coupling steps. Such protective groups are well-known to a person skilled in the art and may be selected from the group consisting of a carboxy,
5 carboxamido, amino, hydroxy, guanidino, sulphide and imidazole moiety.

Without wishing to be bound by any particular theory, it is assumed that the physical-chemical properties of the protected side-chain of the presequence exemplified by lysine are responsible for the observed "structural assisted peptide synthesis" (SAPS)
10 by reducing or eliminating β -sheet formation in the polyalanine sequence.

In accordance with the above observations, another aspect of the present invention relates to a method for the solid phase synthesis of peptides as described above having the further feature that a linker is inserted between the presequence attached to the support
15 and the desired peptide sequence AA_1-AA_n , which enables a selective cleavage of said sequence. Preferably, the linker is optically active. An applicable group of linkers is α -hydroxy and α -amino acids of the general formula



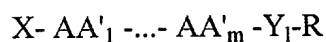
25 wherein X is OH or NH_2 and R^1 and R^2 are independently selected from H, C_{1-3} alkyl, phenyl and substituted phenyl, where the substituents are one or more electron donating substituents chosen among C_{1-3} alkoxy, C_{1-3} alkyl, or two vicinal substituent groups are joined to form a 5 or 6 membered ring together with the carbon atoms to which they are

attached. The most preferred linkers are racemic 4-methoxymandelic acid, (+)-4-methoxymandelic acid, diphenylglycine and glycolic acid.

In a still further embodiment of the invention, a first linker is inserted between the presequence attached to the support and the AA₁-AA_n sequence and a second linker is inserted between the presequence and the solid support with orthogonal cleavage conditions to the first linker enabling a selective cleavage of the second linker, e.g., by means of trifluoroacetic acid (TFA), trifluoromethanesulfonic acid (TFMSA), HBr, HCl, HF or a base such as ammonia, hydrazine, alkoxide or hydroxide to give the desired peptide AA₁-AA_n linked to the presequence by means of said first linker, which is then optionally cleaved from the presequence.

Another important embodiment of the invention relates to agents for use in solid phase synthesis incorporating one or more of the above described features with relation to presequences and linkers.

A first aspect of this embodiment relates to an agent for use in solid phase peptide synthesis which contains a presequence and optionally one or more amino acids from the desired sequence has the general formula



wherein R is a solid support applicable in solid phase peptide synthesis, Y₁ is an amino acid sequence comprising from 3 to 9, preferably from 5 to 7 amino acid residues independently selected from native L-amino acids having a side chain functionality which is protected during the coupling steps and having a propensity factor $P\alpha > 0.57$ and a propensity factor $P\beta \leq 1.10$, e.g., Lys, Glu, Asp, Ser, His, Asn, Arg, Met or Gln, or the

corresponding D-amino acid, AA' is an L- or D-amino acid residue, m is zero or an integer from 1 to 40 and X is hydrogen or an amino protective group.

A second aspect of this embodiment relates to an agent for use in solid phase peptide synthesis which contains a presequence, a preferably optically active cleavage linker and optionally one or more amino acids from the desired sequence and has the general formula



wherein X, AA', m, Y₁ and R are as defined above and L₁ is a preferably optically active linker which enables a selective cleavage of the bond to AA'_m. Preferred linkers are α-hydroxy or α-amino acids as described above.

A third aspect of this embodiment relates to an agent for use in solid phase peptide synthesis which contains a presequence, a first preferably optically active cleavage linker, a second linker enabling cleavage from the support and optionally one or more amino acids from the desired sequence and has the general formula



wherein X, AA', m, Y₁, R and L₁ are as defined above and L₂ is a linker which enables a selective cleavage from the solid support.

A fourth aspect of this embodiment relates to an agent for use in solid phase peptide synthesis which contains a linker enabling cleavage from the support, a presequence and optionally one or more amino acids from the desired sequence and has the general formula



wherein X, AA'₁, m, Y₁, R and L₂ is as defined above.

The specific conditions used in the experiments on which this invention is based are
5 stated below under general procedures.

Generally speaking, apart from the novel and characteristic features related to the
presequence and the novel cleavage linkers, the method according to the invention may be
carried out under traditional conditions for solid phase peptide synthesis described in the
10 literature referred to in the background art. Specifically, Fmoc groups may be
deprotected by means of an amine such as piperidine or diazabicyclo[5,4,0]undec-7-ene
(DBU). Side chain protective groups may be deprotected by means of an acid such as
trifluoroacetic acid (TFA), trifluoromethanesulfonic acid (TFMSA) HBr, HCl or HF.

15 The solid support is preferably selected from functionalized resins such as polystyrene,
polyacrylamide, polyethyleneglycol, cellulose, polyethylene, latex or dynabeads.
If desired, C-terminal amino acids may be attached to the solid support by means of a
common linker such as 2,4-dimethoxy-4'-hydroxy-benzophenone, 4-(4-hydroxy-
methyl-3-methoxyphenoxy)-butyric acid (HMPB), 4-hydroxymethylbenzoic acid,
20 4-hydroxymethylphenoxyacetic acid (HMPA), 3-(4-hydroxymethylphenoxy)propionic
acid or p-[(R,S)- α [1-(9H-fluoren-9-yl)methoxyformamido]-2,4-dimethoxybenzyl]
-phenoxyacetic acid (AM).

The synthesis may be carried out batchwise or continuously on an automated or semi
25 automated peptide synthesizer.

The individual coupling steps may be performed in the presence of a solvent, e.g.,
selected from acetonitrile, N,N-dimethylformamide (DMF), N-methylpyrrolidone

(NMP), dichloromethane (DCM), trifluoroethanol (TFE), ethanol, methanol, water, mixtures of the mentioned solvents with or without additives such as perchlorate or ethylenecarbonate.

- 5 The individual couplings between two amino acids, an amino acid and the earlier formed peptide sequence or a peptide fragment and the earlier formed peptide sequence may be carried out according to usual condensation methods such as the azide method, mixed acid anhydride method, symmetrical anhydride method, carbodiimide method, active ester method such as pentafluorophenyl(Pfp), 3,4-dihydro-4-oxobenzotriazin-3-yl (Dhbt),
10 benzotriazol-1-yl (Bt), 7-azabenzotriazol-1-yl (At), 4-nitrophenyl, N-hydroxysuccinic acid, imidoesters (NHS), acid chlorides, acid fluorides, *in situ* activation by O-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU), O-(benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), O-(benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU), or
15 benzotriazolyl-oxy-tris-(dimethylamio)-phosphonium hexafluorophosphate (BOP).

- The formed peptide may be cleaved from the support by means of an acid such as trifluoroacetic acid (TFA), trifluoromethanesulfonic acid (TFMSA), hydrogen bromide (HBr), hydrogen chloride (HCl), hydrogen fluoride (HF) or a base such as ammonia,
20 hydrazine, an alkoxide or a hydroxide. Alternatively, the peptide is cleaved from the support by means of photolysis.

- In the embodiment, where a linker is inserted between the presequence attached to the support and the AA₁-AA_n sequence which enables a selective cleavage of said sequence,
25 said cleavage may be made by means of an acid such as trifluoroacetic acid (TFA), trifluoromethanesulfonic acid (TFMSA), hydrogen bromide (HBr), hydrogen chloride (HCl), hydrogen fluoride (HF) or a base such as ammonia, hydrazine, an alkoxide or a hydroxide.

EXAMPLES

Abbreviations

5	AM,	p-[(R,S)- α [1-(9H-fluoren-9-yl)methoxyformamido]-2,4-dimethoxybenzyl] -phenoxyacetic acid
	At,	7-azabenzotriazol-1-yl
	Boc,	tert. butyloxycarbonyl
	BOP,	benzotriazolyl-oxy-tris-(dimethylamino)-phosphonium hexafluorophosphate
10	Bpoc,	biphenylpropyloxycarbonyl
	Bt,	benzotriazol-1-yl
	tBu,	tert. butyl
	DBU,	diazabicyclo[5,4,0]undec-7-ene
15	Ddz,	3,5-dimethoxyphenylisopropyloxycarbonyl
	DCC,	dicyclohexylcarbodiimide
	DCM,	dichloromethane
	DIC,	diisopropylcarbodiimide
	DIEA,	N,N-diisopropylethylamine
20	DMAP,	4-(N,N-dimethylamino)pyridine
	Dhbt-OH,	3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine
	DMF,	N,N-dimethylformamide
	Dts,	dithiasuccinyl
	EDT,	ethanedithiol
25	FAB,	fast atom bombardment
	Fmoc,	9-fluorenylmethyloxycarbonyl
	HATU,	O-(7-azabenzotriazol-1-yl)-1,1,3,3- tetramethyluronium hexafluorophosphate

	HBTU,	O-(benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
	HMPA,	4-hydroxymethylphenoxyacetic acid
	HMPB,	4-(4-hydroxymethyl-3-methoxyphenoxy)-butyric acid
5	HObt,	1-hydroxybenzotriazole
	HOAt,	1-hydroxy-7-azobenzotriazole
	HPLC,	high pressure liquid chromatography
	MCPS,	multiple column peptide synthesis
	MHC,	major histocompatibility complex
10	MMa,	4-methoxymandelic acid
	NMR,	nuclear magnetic resonance
	NHS,	N-hydroxy-succinic acid imido ester
	NMP,	N-methylpyrrolidone
	NPS,	nitrophenylsulfenyl
15	Mtr,	4-methoxy-2,3,6-trimethylphenylsulfonyl
	PAM,	phenylacetamidomethyl
	Pbf,	2,2,4,6,7-pentamethyl-2H-benzofuran-5-sulfonyl
	PEG-PS,	polyethyleneglycol grafted on polystyrene
	PepSyn Gel,	polydimethylacrylamide resin functionalized with sarcosine methylester
20		
	PepSyn K,	Kieselguhr supported polydimethylacrylamide resin functionalized with sarcosine methylester
	Pfp,	pentafluorophenyl
	Pmc,	2,2,5,7,8-pentamethylchorman-6-sulfonyl
25	Poc,	phenylisopropylloxycarbonyl
	TBTU,	O-(benzotriazol-1-yl)-1,1,3,3 tetramethyluronium tetrafluoroborate
	TFA,	trifluoroacetic acid
	TFE,	trifluorethanol

TFMSA, trifluoromethanesulfonic acid
Tmz, 2,4,5-tetramethylbenzyloxycarboxyl

General Procedures: Peptide Synthesis

5

Apparatus and synthetic strategy

Peptides were synthesized either batchwise in a polyethylene vessel equipped with a polypropylene filter for filtration or in the continuous-flow version of the polyamide
10 solid-phase method (Dryland, A. and Sheppard, R.C. (1986) J. Chem. Soc. Perkin Trans. I, 125 - 137) on a fully automated peptide synthesizer (Cameron, L., Meldal, M. and Sheppard, R.C (1987), J. Chem. Soc. Chem. Commun. 270-272) using 9-fluorenylmethyloxycarbonyl (Fmoc) or tert. Butyloxycarbonyl, (Boc) as N- α -amino protecting group and suitable common protection groups for side-chain functionalities.

15

Solvents

Solvent DMF (N,N-dimethylformamide, Riedel de-Häen, Germany) was purified by passing it through a column packed with a strong cation exchange resin (Lewatit S 100
20 MB/H strong acid, Bayer AG Leverkusen, Germany) and analyzed for free amines prior to use by addition of 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine(Dhbt-OH) giving rise to a yellow color (Dhbt-0⁻ anion) if free amines are present. Solvent DCM, (dichloromethane, analytical grade, Riedel de-Häen, Germany) was used directly without purification.

25

Amino acids

Fmoc-protected amino acids and corresponding pentafluorophenyl (Pfp) esters were purchased from MilliGen, UK, NovaBiochem, Switzerland and Bachem, Switzerland, and the Dhbt-esters from NovaBiochem, Switzerland in suitable side-chain protected forms. Bocprotected amino acids were purchased from Bachem, Switzerland.

5

Coupling reagents

Coupling reagent diisopropylcarbodiimide (DIC) was purchased from Riedel de-Häen, Germany and distilled prior to use, dicyclohexylcarbodiimide (DCC) was purchased from Merck-Schuchardt, München, Germany, and purified by distillation. O-Benzotriazolyl-N,N,N',N'-tetramethyluronium tetrafluoroborate (TBTU) was purchased from PerSeptive Biosystems GmbH Hamburg, Germany.

10

Linkers

15

Linkers HMPA, Novabiochem, Switzerland; 4-hydroxymethylbenzoic acid, Novabiochem; 4-methoxymandelic acid, Aldrich, Germany; HMPB, Novabiochem; AM, Novabiochem; 3-(4-hydroxymethylphenoxy)propionic acid, Novabiochem, was coupled to the resin as a preformed 1-hydroxybenzotriazole (HObt) ester generated by means of DIC. Racemic 4-methoxymandelic acid (98% pure, Aldrich, Germany) was used directly as linker, or as described below, resolved by treatment with (+)-cinchonine (85% pure, Aldrich, Germany).

20

Resolution of (+/-)-4-methoxymandelic acid (McKenzie, A., Pirie, D.J.C. (1936),

25

Berichte 69, 868, & Knorr, E. (1904), Berichte 37, 3172)

(+/-)-4-Methoxymandelic acid (10 g, 54.89 mmol; Aldrich, 98%) was dissolved in 500 ml hot water (60-80°C) and the solution was decanted while still warm in order to remove

insoluble impurities. (+)-Cinchonine (16.16 g, 54.89 mmol, Aldrich, 85%, $[\alpha]_D^{20} = +211^\circ$ (litt.: $+228^\circ$) was added to the hot solution in small portions. The solution became clear after 15 min stirring at 60-80°C and was cooled in ice. After 1 h, the precipitate was collected by filtration and dried in a dessicator overnight, yielding 9.9 g of the chinconine salt. The salt was recrystallized from boiling water (80 ml), the solution decanted while still warm and then cooled in ice. The precipitate was collected by filtration after 1 h, washed three times with cold water, and dried in a dessicator overnight yielding 7.84 g (16.45 mmol). The chinconine salt (2 g, 4.2 mmol) was dissolved in 40 ml 2N HCl and immediately extracted with 3x 30 ml diethylether. The ether phase was dried over Na_2SO_4 and evaporated to dryness yielding 0.55 g 4-methoxymandelic acid. The optical purity of the liberated 4-methoxymandelic acid was estimated to 18.5% ($[\alpha]_D^{20} = +27^\circ$). After a second recrystallization of the chinconine salt followed by liberation of the mandelic acid as described above, the optical purity was estimated to 69.0 % ($[\alpha]_D^{20} = +100.8^\circ$). A third recrystallization resulted in an optical purity of 95.8% ($[\alpha]_D^{20} = +140.0^\circ$).

Solid supports

Peptides synthesized according to the Fmoc-strategy were synthesized on three different types of solid support using 0.05 M or higher concentrations of Fmoc-protected activated amino acid in DMF: 1) PEG-PS (polyethyleneglycol grafted on polystyrene; TentaGel S NH_2 resin, 0.27 mmol/g, Rapp Polyinere, Germany or NovaSyn TG resin, 0.29 mmol/g, Novabiochem, Switzerland); 2) PepSyn Gel (polydimethylacrylamide resin functionalized with sarcosine methylester, 1.0 mmol/g; MilliGen, UK). 3) PepSyn K (Kieselguhr supported polydimethylacrylamide resin functionalized with sarcosine methylester 0.11 mmol/g; MilliGen, UK).

Peptides synthesized according to the Boc-strategy were synthesized on a Merrifield-resin (polystyrene-divinylbenzene) with the first amino acid attached (Novabiochem, Switzerland).

5 *Catalysts and other reagents*

Diisopropylethylamine (DIEA) was purchased from Aldrich, Germany, and ethylenediamine from Fluka, Switzerland, piperidine from Riedel-de H  en, Frankfurt, Germany. 4-(N,N-dimethylamino)pyridine (DMAP) was purchased from Fluka, Switzerland and used as a catalyst in coupling reactions involving symmetrical anhydrides. 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine (Dhbt-OH) was obtained from Fluka, Switzerland, and 1-hydroxybenzotriazole (HOBT) from NovaBiochem, Switzerland.

15 *Coupling-procedures*

The first amino acid was coupled as a symmetrical anhydride in DMF generated from the appropriate N-  -protected amino acid and DIC. The following amino acids were coupled as Pfp- or Dhbt-esters or as preformed HOBT esters made from appropriate N-  -protected amino acids and HOBT by means of DIC or TBTU in DMF. In the case of Fmoc, all acylations were checked by the ninhydrin test performed at 80  C in order to prevent Fmoc deprotection during the test (Larsen, B.D., and Holm, A. (1994), Int. J. Peptide & Protein Res. 43,1-9).

25 *Deprotection of the N-  -amino protecting group*

Deprotection of the Fmoc group was performed by treatment with 20% piperidine in DMF (1x3 and 1x7 min when synthesized batchwise) or by flowing the deprotection

solvent through the resin (10 min, flow rate 1 ml/min using continuous flow synthesis), followed by wash with DMF until no yellow color (Dhbt-O-) could be detected after addition of Dhbt-OH to the drained DMF.

- 5 Deprotection of the Boc group was performed by treatment with 50% TFA in DCM (v/v) 1x1.5 min and 1x20 min followed by wash 6x9 min each with DCM, neutralization with 10% triethylamine in DCM (v/v) 2x1.5 min each, followed by 6x9 min wash with DCM.

10 *Cleavage of peptide from resin with acid*

Peptides were cleaved from the resins by treatment with 95% trifluoroacetic acid (TFA, Halocarbon Products Corporation, U.S.A.; Biesterfeld & Co. Hamburg, Germany)-water v/v at r.t. for 2 h. The filtered resins were washed with 95% TFA-water and filtrates and washings were evaporated under reduced pressure. The residue was washed with ether and freeze dried from acetic acid-water. The crude freeze dried product was analyzed by high-performance liquid chromatography (HPLC) and identified by matrix assisted laser desorption ionization time of flight mass spectrometry (MALDI TOF MS) or by electrospray ionization mass spectrometry (ESMS).

20

Cleavage of peptide from resin with base

The dried resin (1 g) was treated with 1M sodium hydroxide (10 ml) at 4°C and left for 15 min at r.t. The resin was filtered into a flask containing 10% aq. acetic acid. The peptide was isolated by lyophilization and submitted to gel filtration.

25

Cleavage of peptide from resin with TFMSA

The dried resin (250 mg) was placed in a round-bottomed flask with a stirring bar. Thioanisole/ethanedithiol 2:1, 750 μ l) was added, the mixture chilled in ice, 5 ml TFA was added and the mixture was stirred for 5-10 min. TFMSA (500 μ l) was added dropwise and the reaction continued at r.t. for 30-60 min. The peptide was precipitated after
5 addition of ether.

Deprotection of side chain protective groups

The side chains were generally deprotected simultaneously with the cleavage of the
10 peptide from the resin.

Preformed HObt-ester

Method a. 3 eq. N- α -amino protected amino acid was dissolved in DMF together with 3
15 eq. HObt and 3 eq. DIC. The solution was left at r.t. for 10 minutes and then added to the resin, which had been washed with a solution of 0.2% Dhbt-OH in DMF prior to the addition of the preactivated amino acid.

Method b. 3 eq. N- α -amino protected amino acid was dissolved in DMF together with 3
20 eq. HObt, 3 eq. TBTU and 4.5 eq. DIEA. The solution was left at r.t. for 5 minutes and then added to the resin.

Preformed symmetrical anhydride

25 6 eq. N- α -amino protected amino acid was dissolved in DCM and cooled to 0 $^{\circ}$ C. DCC (3 eq.) was added and the reaction continued for 10 min. The solvent was removed *in*

vacuo and the remains dissolved in DMF. The solution was filtered and immediately added to the resin followed by 0.1 eq. of DMAP.

Estimation of the coupling yield of the first N- α - amino protected amino acid

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3 - 5 mg dry Fmoc-protected peptide-resin was treated with 5 ml 20% piperidine in DMF for 10 min at r.t. and the UV absorption for the dibenzofulvenepiperidine adduct was estimated at 301 nm. The yield was determined using a calculated extension coefficient ϵ_{301} based on a Fmoc-Ala-OH standard.

10

In case of Boc-protection, the coupling was estimated according to the ninhydrin-method after removal of the Boc-group (Sarin, V.Y., Kent, S.B.H., Tam, J.P. and Merrifield, R.B. (1981), Anal. Biochem. 117, 147-157).

15

Peptide synthesis on PepSyn K resin

Dry PepSyn K (ca 500 mg), was covered by ethylenediamine and left at r.t. overnight.

The resin was drained and washed with DMF 10 x 15 ml, 5 min each. After draining, the resin was washed with 10% DIEA in DMF v/v (2 x 15 ml, 5 min each) and finally

20

washed with DMF until no yellow color could be detected by addition of Dhbt-OH to the drained DMF. 3 eq. HMPA, 3 eq. HOBT and 3 eq. DIC were dissolved in 10 ml DMF and left for activation for 10 min, after which the mixture was added to the resin and the coupling continued for 24 h. The resin was drained and washed with DMF (10 x 15 ml, 5 min each), and the acylation was checked by the ninhydrin test. The first amino acid was

25

coupled as the side chain protected preformed symmetrical anhydride (see above), and the coupling yields estimated as described above. It was in all cases better than 70%. The synthesis was then continued either as "*continuous-flow*" or as "*batchwise*" as described below.

Continued peptide synthesis on PepSyn K using continuous-flow technique

The resin (ca. 500 mg) with the first amino acid attached was placed in a column
5 connected to the fully automated peptide synthesizer. The Fmoc group was deprotected
as described above. The remaining amino acids according to the sequence were coupled as
Fmoc protected, if necessary, side chain protected, Pfp esters (3 eq.) with the addition of
Dhbt-OH (1 eq.). The end-point of each coupling was determined automatically by
10 monitoring the disappearance of the yellow color of the Dhbt-OH anion
spectrophotometrically. After completed synthesis, the peptide resin was washed with
DMF (10 min flow rate 1 ml/min), DCM (3x5 ml, 3 min each) and finally diethyl ether
(3x5 ml each), removed from the column and dried *in vacuo*.

Continued batchwise peptide synthesis on PePSyn

15 The resin (ca. 500 mg) with the first amino acid attached was placed in a polyethylene
vessel equipped with a polypropylene filter for filtration, and the Fmoc-group
deprotected as described above. The remaining amino acids according to the sequence
were coupled as preformed Fmoc-protected, if necessary, side chain protected, HOBT
20 esters (3 eq.) in DMF (5 ml) prepared as described above. The couplings were continued
for 2 h unless otherwise specified. Excess reagent was then removed by DMF washing
(12 min, flow rate 1 ml/min) All acylations were checked by ninhydrin test performed at
80 °C. After completed synthesis, the peptide-resin was washed with DMF (10 min,
flow rate 1 ml/min), DCM and finally diethyl ether (5x5 ml, 1 min each) and finally
25 diethyl ether (5x5 ml, 1 min each) and dried *in vacuo*.

Batchwise Peptide synthesis on PEG-PS

coupling was continued for 24 h. The resin was drained and washed with DMF (5 x 15 ml, 5 min each). The acylation was checked by the ninhydrin test. The first amino acid was coupled as preformed side chain protected symmetrical anhydride as described above. The coupling yields of the first Fmoc-protected amino acids were estimated as described above. It was in all cases better than 70%. The remaining amino acids according to the sequence were coupled as preformed Fmoc-protected, if necessary side chain protected, Hobbt esters (3 eq.) as described above (method a). The couplings were continued for 2 h and, if necessary, double coupled overnight. The resin was drained and washed with DMF (5 x 5 ml, 5 min each) in order to remove excess reagent. All acylations were checked by the ninhydrin test performed at 80°C. The Fmoc group was deprotected as described above. After completed synthesis, the peptide-resin was washed with DMF (3x15 ml, 5 min each), DCM (3x15 ml, 2 min each) and finally diethyl ether (3x15 ml, 2 min each) and dried *in vacuo*.

HPLC conditions

HPLC was performed on a Waters 600 E instrument equipped with a Waters 996 Photodiode array detector with a Waters Radial Pak 8 x 100 mM C₁₈ reversed-phase column. Buffer A was 0.1 vol % TFA in water and buffer B was 90 vol% acetonitrile, 9.9 vol% water and 0.1 vol% TFA. The buffers were pumped through the column at a flow rate of 1.5 ml/min using the gradient: 1. Linear gradient from 0%-70% B (20 min), linear gradient from 70-100% B (1 min) isocratic 100% B (5 min). 2. Isocratic with 0% B (2 min), linear gradient from 0-50% B (23 min), linear gradient from 50-100% B (5 min), isocratic 100% B (5 min).

Mass spectroscopy

Matrix assisted laser desorption ionization time-of-flight (MALDI TOF) mass spectra were obtained on a Fisons TofSpec E instrument. Electrospray ionization mass spectra were obtained on a Finnigan Mat LCQ instrument equipped with an electrospray (ESI) probe (ES-MS).

5

Syntheses of H-(Ala)_n-(Lys)_m-OH Oligomers

To investigate the problems described above with reference to B. D. Larsen, C. Larsen, and A. Holm., Peptides 1990; E. Giralt and D. Andreu, (Eds.), 1991 ESCOM Science Publishers B.V., p. 183-185 & Larsen, B.D., and Holm, A. (1994), Int. J. Peptide &

10 Protein Res. 43,1-9, further, the degree to which the β -sheet formation of the homo oligo-alanine chain may be affected by inclusion of a shorter peptide sequence, a presequence, in the chain at the C-terminus was addressed. This question is associated with the fact that protein structures and polypeptide sequences may have stretches of well defined structures dependent on the amino acids in the sequence and of preceding
15 amino acids. As mentioned earlier, Chou and Fasman's investigations (Chou, P.Y. and Fasman, G.D. (1978), Ann. Rev. Biochem. 47, 251-276.) on protein structures have led to recognition of classes of amino acids which are defined as predominantly α -helix inducing β -sheet or random coil inducing. Although it *a priori* may be assumed that similar predictions may apply for homo oligo alanines or leucines it was pointed out,
20 however, in Larsen, B.D., and Holm, A. (1994), Int. J. Peptide & Protein Res. 43, 1-9. that Chou and Fasman rules do not predict homo oligoalanine or leucine peptides as β -sheet forming chains. Furthermore, Chou and Fasman's rules cannot be expected to apply during peptide synthesis on a resin and do clearly not apply when side-chain protected amino acids are part of the synthesis.

25

Peptide synthesis of individual peptides

Synthesis of H-Ala₁₀-Lys-OH (SEQ ID NO:3) (comparison)

500 mg Fmoc-Lys(Boc) PepSyn KA resin (0.086 mmol/g) was used for synthesis according to "continuous-flow technique".

- 5 The crude freeze dried product was analyzed by HPLC and found to be a complicated mixture comprising the target peptide (n = 10) as well as deletion peptides corresponding to n = 6, 7, 8, and 9 and deletion peptides with the Fmoc group still attached to the N-terminal where n = 6, 7, 8, and 9 respectively (see Figure 2). The identity of the individual peptides was confirmed by MALDI TOF MS.

10 Synthesis of H-Ala₁₀-Lys₂-OH (SEQ ID NO:4)

500 mg Fmoc-Lys(Boc) PepSyn KA resin (0.086 mmol/g) was used for synthesis according to "continuous-flow technique".

15 The crude freeze-dried product was analyzed by HPLC and found to be homogeneous without deletion and Fmoc-protected sequences. Yield 91.0%. The purity was found to be better than 98% according to HPLC (see Figure 4). The identity of the peptide was confirmed by MALDI TOF MS.

20 Synthesis of H-Ala₁₀-Lys₆-OH (SEQ ID NO:6)

500 mg Fmoc-Lys(Boc) PepSyn KA resin (0.086 mmol/g) was used for synthesis according to the "continuous-flow technique".

25 The crude freeze dried product was analyzed by HPLC and found to be homogeneous without deletion and Fmoc-protected sequences. Yield 90.9%. The purity was found to

be better than 98% according to HPLC (see Figure 6). The identity of the peptide was confirmed by ES MS.

Synthesis of H-Ala₂₀-Lys₃-OH (SEQ ID NO:5)

5

500 mg Fmoc-Ala₁₀-(Lys(Boc))₃ PepSyn KA resin (SEQ ID NO:28) (from the synthesis of H-Ala₁₀-Lys₃-OH) (SEQ ID NO:4) was used for synthesis according to the "continuous-flow technique" and the synthesis was continued.

10 The crude freeze dried product was analyzed by HPLC and found to comprise the target peptide H-Ala_n-Lys₃-OH (SEQ ID NO:30)(n = 20) as well as deletion peptides corresponding to n = 19, 18, 17 and 16 (see Figure 5). Fmoc-protected sequences were not detected. The identity of the peptides were confirmed by ES MS.

Synthesis of H-Ala₂₀-Lys₆-OH (SEQ ID NO:5)

500 mg Fmoc-Lys(Boc) PepSyn KA resin (0.086 mmol/g) was used for synthesis according to the "continuous-flow technique".

20 The crude freeze dried product was analyzed by HPLC and found to be homogeneous without deletion and Fmoc-protected sequences. Yield 91.4%. The purity was found to be better than 98% according to HPLC (see Figure 7). The identity of the peptide was confirmed by ES MS.

Synthesis of H-Ala₂₀-Lys-(Gly-Lys)₂-OH (SEQ ID NO:29)

500 mg Fmoc-Lys(Boc) PepSyn KA resin (0.086 mmol/g) was used for synthesis according to the "continuous-flow technique".

The crude freeze-dried product was analyzed by HPLC. It was found to be better than 98% pure without deletion and Fmoc-protected sequences. Yield 97%. The identity of the peptide was confirmed by ES MS.

5

Synthesis of H-Ala₁₀-Lys-OH (SEQ ID NO:3) using (Lys(Boc))₆ (SEQ ID NO:22) as presequence and HMPA as linker (H-Ala₁₀-Lys(Boc)-OCH₂-PhOCH₂CO-(Lys(Boc))₆-NHCH₂-CH₂NH PepSyn K)(SEQ ID NO:31)

10 500 mg dry PepSyn K (0.1 mmol/g) was covered by ethylenediamine (5 ml) and left at r.t. overnight. The resin was drained and washed with DMF 10 x 15 ml, 5 min each. After draining the resin was washed with 10% DIEA in DMF v/v (2 x 15 ml, 5 min each) and finally washed with DMF until no yellow color could be detected by addition of Dhbt-OH to the drained DMF. The derivatized resin was used for synthesis according to the "continuous-flow technique".

15 The first 6 lysines forming the presequence were coupled as Fmoc-Lys(Boc)-Pfp esters (3 eq.) with the addition of Dhbt-OH (1 eq.). The end-point of each coupling was determined automatically as described above. The Fmoc group was cleaved as described above. After finishing the presequence, 3 eq. HMPA coupled as a preactivated HOBT ester as described above was introduced at the top of the column. The synthesizer was operated in recirculation mode for 2 h and excess of reagent was then removed by DMF washing (12 min, flow rate 1 ml/min). A small resin-sample was removed in order to check the acylation by the ninhydrin test. The next amino acid according to the sequence

20 was coupled as preformed side chain protected symmetrical anhydride as described above and introduced at the top of the column together with (0.1 eq.) DMAP and the synthesizer was operated in recirculation mode for 90 min. Excess reagent was then removed by DMF washing (12 min, flow rate 1 ml/min). A small resin-sample was

removed in order to check the coupling yield, which was estimated as earlier described and found to be 84%. The synthesis was then continued by cleavage of the Fmoc group as described above. The remaining amino acids according to the sequence were coupled as Fmoc-protected, if necessary side chain protected, Pfp esters (3 eq.) with addition of
5 Dhbt-OH (1 eq.). The end-point of each coupling was determined automatically as described above. After completed synthesis, the peptide-resin was washed with DMF (10 min, flow rate 1 ml/min), DCM (3x5 ml, 1 min each) and finally diethyl ether (3x5 ml, 1 min each), removed from the column and dried *in vacuo*.

10 The peptide was cleaved from the resin as described above.

The crude freeze dried product was analyzed by HPLC and found to comprise the target peptide H-Ala_n-Lys-OH (SEQ ID NO:34)(n = 10) as well as deletion peptides corresponding to n = 9, 8, 7 and 6 (see Figure 8). Fmoc-protected sequences were not
15 detected. The identity of the peptides were confirmed by MALDI TOF MS.

Synthesis of H-Ala₁₀-Lys-OH(SEQ ID NO:3) using (+/-)-4- methoxymandelic acid as linker (H-Ala₁₀-Lys(Boc)-OCH-(4-MeOPh)CONHCH₂CH₂NH PepSyn K resin) (SEQ ID NO:32)

500 mg dry PepSyn K (0.1 mmol/g) was treated with ethylenediamine as described above. The derivatized resin was used for synthesis according to the "continuous-flow technique". 10 eq. (+/-)-4-methoxymandelic acid, 10 eq. HOBT and 10 eq. DIC dissolved in 5 ml DMF, preactivated for 10 min were introduced at the top of the column and the
20 synthesizer was operated in recirculation mode for 2 h. Excess of reagent was then removed by DMF washing (12 min, flow rate 1 ml/min). A small resin-sample was removed in order to check the acylation by the ninhydrin test. The first amino acid according to the sequence was coupled as Fmoc protected and side chain protected

preformed symmetrical anhydride as described above and introduced at the top of the column together with (0.1 eq.) DMAP and the synthesizer was operated in recirculation mode for 90 min. Excess reagent was then removed by DMF washing (12 min, flow rate 1 ml/min). A small resin-sample was removed in order to check the coupling yield, which
5 was estimated as described above and found to be 75%. The synthesis was then continued by cleavage of the Fmoc group as earlier described. The remaining amino acids according to the sequence were coupled as Fmoc-protected Pfp esters (3 eq.) with the addition of Dhbt-OH (1 eq.). The end-point of each coupling was determined automatically as described above. After completed synthesis, the peptide-resin was
10 washed with DMF (10 min, flow rate 1 ml/min), DCM (3x5 ml, 1 min each) and finally diethyl ether, removed from the column and dried *in vacuo*.

The peptide was cleaved from the resin as earlier described and lyophilized from acetic acid-water. The crude freeze dried product was analyzed by HPLC and found to
15 comprise the target peptide H-Ala_n-Lys-OH (n = 10) as well as deletion peptides corresponding to n = 9, 8, 7 and 6 (Figure 9). The identity of the peptides were confirmed by MALDI TOF MS.

Synthesis of H-Ala₁₀-Lys-OH(SEQ ID NO:3) using (Lys(Boc))₆(SEQ ID NO:22) as
20 presequence and (+/-)-4-methoxymandelic acid as linker (H-Ala₁₀-Lys(Boc)-OCH-(4MeOPh)CO-(Lys(Boc))₆-NHCH₂CH₂NH PepSyn K resin)(SEQ ID NO:33)

500 mg dry PepSyn K (0.1 mmol/g) was treated with ethylenediamine as described above. The derivatized resin was used for synthesis according to the "continuous-flow
25 technique". The first 6 Lysines forming the presequence were coupled as Fmoc-protected and side chain protected Pfp esters (3 eq.) with the addition of Dhbt-OH (1 eq.). The end-point of each coupling was determined automatically as described above. The Fmoc group was cleaved as described above. After completed synthesis, of the presequence, 10

eq. (+/-)-4-methoxymandelic acid was coupled as preactivated HOBT-ester as earlier described and introduced at the top of the column. The synthesizer was operated in recirculation mode for 2 h and excess reagent was then removed by DMF washing (12 min, flow rate 1 ml/min). A small resin-sample was removed in order to check the acylation by the ninhydrin test. The next amino acid according to the sequence was coupled as Fmoc protected and side chain protected preformed symmetrical anhydride as earlier described and introduced at the top of the column together with (0.1 eq.) DMAP. The synthesizer was operated in recirculation mode for 90 min and excess reagent was then removed by DMF washing (12 min, flow rate 1 ml/min). A small resin-sample was removed in order to check the coupling yield, which was estimated as described above and found to be 68%. The synthesis was then continued by cleavage of the Fmoc group as earlier described. The remaining amino acids according to the sequence were coupled as Fmoc protected Pfp esters (3 eq.) with the addition of Dhbt-OH (1 eq.). The end-point of each coupling was determined automatically as described above. After completed synthesis, the peptide-resin was washed with DMF (10 min, flow rate 1 ml/min), DCM (3x5 ml min, flow rate 1 ml/min) and finally diethyl ether (3x5 ml min, flow rate 1 ml/min), removed from the column and dried *in vacuo*. The peptide was cleaved from the resin as earlier described and freeze dried from acetic acid-water. The crude freeze dried product was analyzed by HPLC and found to comprise the target peptide H-Ala_n-Lys-OH (SEQ ID NO:34)(n = 10) as well as deletion peptides corresponding to n = 9, 8, 7 and 6. The amount of deletion peptides was found to be significantly reduced compared to the synthesis of SEQ ID NOS: 31 and 33 as described above (see Figure 9). Fmoc-protected sequences were not detected. The identity of the peptides were confirmed by MALDI TOF MS.

Synthesis of H-Ala₁₀-Lys-OH (SEQ ID NO:3) using (Lys(Boc))₆ (SEQ ID NO:22) as presequence and (+)-4-methoxymandelic acid as linker (H-Ala₁₀-Lys(Boc)-OCH-4-MeOPh)Co(Lys(Boc))₆-NHCH₂CH₂NH. PepSyn K resin)(SEQ ID NO:33)

Dry PepSyn K (ca 500 mg, 0.1 mmol/g) was placed in a polyethylene vessel equipped with a polypropylene filter for filtration and treated with ethylenediamine as earlier described. The first 6 lysines forming the presequence were coupled as Fmoc-protected and side chain protected Pfp esters (3 eq.) with the addition of Dhbt-OFI (1 eq.). The acylations were checked by the ninhydrin test performed at 80°C as described above. The Fmoc group was deprotected as described above. After finishing the presequence, the deprotected peptide-resin was reacted with 10 eq. (+)-4-methoxymandelic acid as a preactivated Hobt-ester as described above (resolved as described above, 95.8% optical purity) and the coupling was continued for 24 h. Excess reagent was removed by DMF washing (12 min, flow rate 1 ml/min). The acylation was checked by the ninhydrin test. The next amino acid according to the sequence was coupled as Fmoc protected and side chain protected preformed symmetrical anhydride as described above and the reaction was continued for 2 h. Excess reagent was then removed by DMF washing (12 min flow rate 1 ml/min). A small resin-sample was removed in order to check the coupling yield, which was estimated as described above, and found to be 66%. The synthesis was then continued by cleavage of the Fmoc group as described above.

The first alanine was coupled as a Fmoc-protected Pfp ester (3 eq.) with the addition of Dhbt-OH (1 eq.) in DMF (2 ml) for 2 h. Excess reagent was then removed by DMF washing (12 min, flow rate 1 ml/min) and the acylation was checked by the ninhydrin test performed at 80°C as described above. The Fmoc group was then removed by treatment with 2% piperidine in DMF v/v (1 min, flow rate 1 ml/min) followed by flushing with DMF (10 sec, flow rate 10 ml/min), flowing with 0.2% Dhbt-OH in DMF (20 min, flow rate 1 ml/min) and finally washing with DMF (2x5 ml, 1 min each). The following Fmoc-protected alanine was coupled immediately as a Pfp ester (3 eq.) with the addition of 1 eq. Dhbt-OH in DMF (2 ml) for 2 h. The acylation was checked by the ninhydrin test performed as described above. The remaining amino acids according to the sequence

were coupled as Fmoc-protected Pfp esters (3 eq.) with the addition of Dhbt-OH (1 eq.) in DMF (2 ml). Excess reagent was removed by DMF washing (12 min flow rate 1 ml/min) and acylations were checked by the ninhydrin test performed 80 °C as described above. The Fmoc group was deprotected as described above. After completed synthesis, the peptide- resin was washed with DMF (10 min, flow rate 1 ml/min), DCM (3x5 ml, 1 min each), diethyl ether (3x5 ml, 1 min each) and dried *in vacuo*.

The peptide was cleaved from the resin as described above and freeze dried from glacial acetic acid. The crude freeze dried product was analyzed by HPLC and found to be homogeneous without deletion and Fmoc-protected sequences.

Synthesis of H-Ala₁₀-Lys-OH (SEQ ID NO:3) using (Glu(OtBu))₆ (SEQ ID NO:36) as presequence and (+)-4 methoxymandelic acid as linker (H-Ala₁₀-Lvs(Boc)-OCH(4-MeOPh)CO-(Glu(OtBu))₆-NHCH₂CH₂NH PepSyn K resin)(SEQ ID NO:35)

Dry PepSyn K (ca 500 mg) was placed in a polyethylene vessel equipped with a polypropylene filter for filtration and treated with ethylenediamine as described above. The first 6 glutamic acids forming the presequence, were coupled as Fmoc-Glu(OtBu)-Pfp esters (3 eq.) with the addition of Dhbt-OH (1 eq.). The acylations were checked by the ninhydrin test performed at 80°C as described above. The Fmoc group was deprotected as described above. After finishing the presequence, 10 eq. (+)-4-methoxymandelic acid (resolved as described above, 95.8% optical purity) was coupled as a preactivated HOBT-ester as described above. The coupling was continued for 24 h and excess reagent was then removed by DMF washing (12 min flow rate 1 ml/min). The acylation was checked by the ninhydrin test. The next amino acid according to the sequence was coupled as Fmoc protected and side chain protected preformed symmetrical anhydride as described above catalyzed by DMAP (0.1 eq.) and the reaction was continued for 2 h. Excess reagent was removed by DMF washing (12 min flow rate,

1 ml/min). A small resin-sample was removed in order to check the coupling yield, which was estimated as described above and the yield found to be 75%. The synthesis was then continued by cleavage of the Fmoc group as described above.

- 5 The first alanine was coupled as a Fmoc-protected Pfp ester (3 eq.) with the addition of Dhbt-OH (1 eq.) in DMF (2 ml) for 2 h. Excess reagent was then removed by DMF washing (12 min flow rate 1 ml/min) and the acylation was checked by the ninhydrin test performed at 80 °C as described above. The Fmoc group was then removed by treatment with 2% piperidine in DMF v/v (1 min, flow rate 1 ml/min) followed by flushing with
- 10 DMF (10 sec, flow rate 10 ml/min), flowing with 0.2% Dhbt-OH in DMF (20 min, flow rate 1 ml/min) and finally washing with DMF (2x5 ml, 1 min each). The following Fmoc-protected alanine was coupled immediately as a Pfp ester (3 eq.) with the addition of 1 eq Dhbt-OH in DMF (2 ml) for 2 h. The acylation was checked by the ninhydrin test performed as described above. The remaining amino acids according to the sequence
- 15 were coupled as Fmoc-protected Pfp esters (3 eq.) with the addition of Dhbt-OH (1 eq.) in DMF (2 ml). Excess reagent was removed by DMF washing (12 min flow rate 1 ml/min) and acylations were checked by the ninhydrin test performed 80 °C as described above. The Fmoc group was deprotected as described above. After completed synthesis, the peptide-resin was washed with DMF (10 min, flow rate 1 ml/min), DCM (3x5 ml, 1
- 20 min each), diethyl ether (3x5 ml, 1 min each) and dried *in vacuo*.

The peptide was cleaved from the resin as described above and freeze dried from glacial acetic acid. The crude freeze dried product was analyzed by HPLC and found to be homogeneous without deletion and Fmoc-protected sequences.

25

Results and Discussion

In the basic studies, the synthesis of H-(Ala)_n- (Lys)_m-OH (SEQ ID NO:12), where (Lys)_m represent the presequence with m equal to 1, 3 and 6 was investigated. A continuous-flow version of the polyamide solid-phase method (Dryland, A. and Sheppard, R.C.(1986) J. Chem. Soc. Perkin Trans. I, 125-137.) on a fully automated peptide synthesizer developed was used as previously described (Cameron, L., Meldal, M. and Sheppard, R.C (1987), J. Chem. Soc. Chem. Commun. 270-272.) with DMF as solvent and 3 times excess of Fmoc-alanine and Fmoc-lysine(tBoc)-pfp esters, respectively and standard Fmoc-deprotection with 20% piperidine in DMF for 10 minutes. Coupling times were monitored with Dhbt-OH which is deprotonated to the yellow Dbht-O⁻ anion when un-acylated amino groups are still present and disappearance of the yellow colour marks the end-point of the synthesis. After cleavage of the peptide from the resin with 95% aqueous TFA, the product was washed with ether and analyzed by HPLC.

In the case of synthesis of H-(Ala)_n-Lys-OH on a polyamide polymerized kieselguhr matrix (PepSyn K), no problems were observed until n = 5, but approximately 20-25% Fmoc-protected peptide was still present after standard deprotection with piperidine (20% piperidine in DMF) with n = 6. When continuing the synthesis to n = 10, a relatively complicated mixture was obtained comprising the target peptide (n = 10) as well as deletion peptides corresponding to n = 6, 7, 8, and 9 and deletion peptides with the Fmoc group still attached to the N-terminal where n = 6, 7, 8, and 9 respectively (Figure 2). This mixture was identified by FAB MS after HPLC separation of the single components. Failure sequences or partial deprotection with n = 2 - 5 or incomplete deprotection of the target peptide (n = 10) was not observed, thus confining the problems to a given stretch of the homo-oligopeptide chain. This type of difficult aminoacylations and incomplete deprotections can therefore be referred to as non-random in contrast to random difficulties which are due only to common steric problems. Although experimental conditions were varied, e.g. resin-type, deprotection time, solvents, addition

of chaotropes, problems still persisted although heating to 50°C had an optimal effect on incomplete Fmoc-deprotection.

In the case of $m = 3$, it was seen from the HPLC trace shown in Figure 4 that the

5 synthesis may be continued to Ala₁₀ (SEQ ID NO:5) without detectable amounts of deletion peptides or incomplete Fmoc-deprotection. However, when continuing the synthesis to Ala₂₀ (SEQ ID NO:14), the chromatogram (Figure 5) shows the presence of a small amount of deletion peptides. The results are even more striking with H-(Ala)_n-(Lys)₆-OH (SEQ ID NO:13), where products without detectable deletion peptides
10 are obtained with both Ala₁₀ (SEQ ID NO:5) (Figure 6) and Ala₂₀ (SEQ ID NO:14) (Figure 7). Furthermore, coupling times are drastically reduced from up to 30 hours to standard coupling times (< 2 hours) in the single steps.

Previous attempts to synthesize the H-Ala₂₀-OH (SEQ ID NO:14) sequence by the
15 Boc-methodology have been made but high levels of deletion and insertion peptides were obtained (Merrifield, R. B., Singer, J. and Chait, B. T. (1988) Anal. Biochem. 174,399-414.). Clearly the presequence Lys₆ (SEQ ID NO:15), which under the prevailing synthesis conditions was fully protected with the tBoc-group, has a most definitive and favourable effect on the structure of the growing peptide chain eliminating
20 the otherwise very severe synthetic problems due to incomplete deprotections and extremely slow couplings. In the case of other homo-oligo presequences, it has been observed that (Glu(tBu))₆ (SEQ ID NO:16), as well as the mixed sequence (Glu(tBu)Lys(tBoc))₃ (SEQ ID NO:17) induces a favourable structure in the poly-alanine chain affording products without deletion peptides.

25 In the above described cases, the peptide sequences have been obtained with a hexa lysine presequence which for certain purposes may be acceptable or even of advantage. Thus, a β -sheet forming sequence may cause severe solubility problems, but in the case of H-

Ala₂₀(Lys)₆-OH (SEQ ID NO:7), the peptide turned out to be soluble in aqueous solutions while H-Ala₁₀-Lys-OH (SEQ ID NO:3) very rapidly precipitated from TFA solutions when diluted with water. In case of (Glu)₆ (SEQ ID NO:23), the solubility provided by the presequence and the multitude of carboxylic acid groups may be utilized for e.g. ELISA, where site-directed attachment to a suitably amino-group activated surface was possible because of facile activation in aqueous solution with e.g. a carbodiimide. However, it has also been considered, how the observations may be utilized for practical peptide synthesis without presence of the presequence in the final product.

The introduction of an optically active linker between (Ala)_n and (Lys)_m was investigated using 4-methoxymandelic acid (MMA) as a possible candidate. This type of linker has supposedly not been used hitherto. The presence of the methoxy group was assumed to facilitate the cleavage of the target peptide from the linker by means of standard scavenger containing TFA solutions. 4-Methoxymandelic acid may be resolved in its optically active forms of which the R-configuration ((+)-configuration) was identical to L-protein amino acids. Two types of experiments have been carried out: (a) synthesis using racemic 4-methoxymandelic acid; (b) synthesis using R-4-methoxymandelic acid (R-MMA). The synthesis scheme is shown in Figure 10 and described above.

In the first case, the sequence resin-Lys(tBoc)₆MMA-Lys-Ala₁₀ (SEQ ID NO:25) was synthesized to give H-Lys-Ala₁₀-OH (SEQ ID NO:26) where the lysine group was introduced to enhance solubility in aqueous solutions for HPLC analysis. The results of the HPLC analysis are shown in Figure 9. A much better product was achieved than for the HMPA-linker (Figure 8) although deletion peptides are present. In case (b) the same peptide was synthesized under identical conditions and a peptide was formed without detectable deletion peptides or incomplete Fmoc-deprotection. These results may be compared to synthesis of H-Lys-Ala₁₀-OH (SEQ ID NO:26) using the construct resin-

MMA-Lys-Ala₁₀ (SEQ ID NO:27) where the presequence Lys(tBoc)₆ (SEQ ID NO:22) was omitted. Significant amounts of deletion peptides are noted.

Syntheses of Difficult Sequences

5

To investigate whether SAPS is a more general phenomenon or if it is confined only to homo-oligopeptides such as the polyalanine sequence, some mixed sequences reputedly known as "difficult sequences" were investigated.

- 10 The synthesis of H-VQAAIDYING-OH (SEQ ID NO:8), Acyl Carrier Protein (ACP) 65-74, is a well known difficult synthesis and has been used as a model reaction in a number of cases (Cameron et al., (1987), J. Chem. Soc. Chem. Commun. 270-272).

Peptide Synthesis

- 15 Synthesis of Acyl Carrier Protein (ACP) 65-74.

H-Val-Gln-Ala-Ala-Ile-Asp-Tyr-Ile-Asn-Gly-OH (SEQ ID NO:8) (comparison)

500 mg Fmoc-Gly PepSyn KA resin (0.074 mmol/g) was used for synthesis according to "continuous-flow technique".

20

The crude freeze dried product was analyzed by HPLC and found to contain the target molecule accompanied by ca. 16% of the des-Val peptide (see Figure 11). The identity of the peptides was confirmed by MALDI TOF MS.

- 25 Synthesis of Acyl Carrier Protein (ACP)

65-74, H-Val-Gln-Ala-Ala-Ile-Asp-Tyr-Ile-Asn-Gly-Lys₆-OH
(SEQ ID NO:9), using the presequence (Lys(Boc)₆ (SEQ ID NO:22))

500 mg Fmoc-Lys(Boc) PepSyn KA resin (0.086 mmol/g) was used for synthesis according to the "continuous-flow technique".

The crude freeze dried product was analyzed by HPLC and found to contain the target peptide in high purity (~95%) with a significantly reduced amount of the des-Val peptide. The identity of the peptide was confirmed by MALDI TOF MS (Figure 12).

Synthesis of H-Val-Asn-Val-Asn-Val-Gln-Val-Gln-Val-Asp-Lys₆-OH (SEQ ID NO:19), using the presequence (Lys (Boc))₆ (SEQ ID NO:22)

500 mg Fmoc-Lys(Boc) PepSyn KA resin (0.086 mmol/g) was used for synthesis according to the "continuous-flow technique".

The crude freeze dried product was analyzed by HPLC and found to be better than 90% pure without deletion and Fmoc-protected sequences. Yield 100%. The identity of the peptide was confirmed by MALDI TOF MS.

Peptide-synthesis-of Tyr-Gly-Gly-Phe-Leu-Lys₆-OH (SEQ ID NO:37) on NovaSyn TentaGel

Dry NovaSyn TG resin (0.29 mmol/g, 250 mg) was placed in a polyethylene vessel equipped with a polypropylene filter for filtration and treated as described under "batchwise peptide synthesis on PEG-PS" until finishing the presequence Lys₆. The following amino acids forming the Leu-enkephalin sequence were coupled as preformed Fmoc-protected HOBT esters (3 eq.) in DMF (5 ml) generated by means of DIC. Before each of the last five couplings a solution of Dhbt-OH (80 mg in 25ml) is added to follow the disappearance of the yellow color as the coupling reaction proceeds. When the yellow color was no longer visible the couplings were interrupted by washing the resin with

DMF (5 x 5 ml, 5 min each). The acylations were then checked by the ninhydrin test performed at 80°C as earlier described. After completed synthesis, the peptide-resin was washed with DMF (3x5 ml, 1 min each), DCM (3x5 ml, 1 min each), diethyl ether (3x5 ml, 1 min each) and dried *in vacuo*.

5

The peptide was cleaved from the resin as described above and freeze dried from acetic acid. The crude freeze dried product was analyzed by HPLC and found to be homogeneous without deletion and Fmoc-protected sequences. The purity was found to be better than 98% and the identity of the peptide was confirmed by ESMS. Yield 84%.

10

Results and Discussion

As described above, the difficult sequence HVQAAIDYING-K₆-OH (SEQ ID NO:9) was synthesized using the presequence (Lys(tBoc))₆ (SEQ ID NO:21) attached at the C-terminus on a pepsyn K. The synthesis proceeded to give a product with the correct molecular weight in high purity and a significantly reduced amount of des-Val peptide.

15

Another difficult sequence is reported to be HVNVNVQVQVD-OH (SEQ ID NO:18) which has been synthesized on a variety of flow resins (Rapp polymer, PEG-PS, Pepsyn K, PEGA 1900/300, PEGA 800/130 and PEGA 300/130) in all cases accompanied by considerable glutamine preview already from Val¹ except when using a PEGA 1900/300 resin (Meldal, M. (1993), Peptides 1993; Schneider, C.H. and Eberle, A.N. (Eds) 1993 ESCOM Science Publishers B,V., p. 61-62). In accordance with the invention, the synthesis of H-VNVNVQVQVDK₆-OH (SEQ ID NO:19) proceeded to give a product with the correct molecular weight. Deletion peptides were not detected in the spectrum.

20

25

In order to verify another important aspect of the present invention, viz. the reduced coupling times obtained by introducing a presequence at the C-terminal part of desired

peptide the coupling times of the individual amino acids in the synthesis of enkephalin, H-Tyr-Gly-Gly-Phe-Leu-OH (SEQ ID NO:20), have been monitored with and without the presequence (Lys(tBoc))₆ (SEQ ID NO:21). These results are shown in Table 2.

Table 2.

Amino acid	Peptide-resin	Coupling time	
		With pre-sq.	Without pre-sq
	Pre-sequence(Lys(tBoc)) ₆ (SEQ ID NO:22)		
Fmoc-Leu-OH	H-Lys(Boc) ₆ -HMPA-R	< 2 min	-
	(SEQ ID NO:22)		
Fmoc-Phe-OH	H-Leu-Lys(Boc) ₆ -HMPA-R	< 5 min	< 120 min
	(SEQ ID NO:38)		
Fmoc-Gly-OH	H-Phe-Leu-Lys(BOC) ₆ -HMPA-R	< 2 min	< 60 min
	(SEQ ID NO:39)		
Fmoc-Gly-OH	H-Gly-Phe-Leu-Lys(tBOC) ₆ -HMPA-R	< 2 min	< 60 min
	(SEQ ID NO:40)		
Fmoc-Tyr-OH	H-Gly-Gly-Phe-Leu-Lys(tBoc) ₆ -HMPA-R	< 1 min	< 40 min
	(SEQ ID NO:41)		
	H-Tyr-Gly-Gly-Phe-Leu-Lys(tBoc) ₆ -HMPA-R		
	(SEQ ID NO:42)		

R= NovaSyn TG derivatized with the linker 4-hydroxymethyl-phenoxy acetic acid (HMPA) (Subst.:0.29 mmol/g). Coupling times are based on the Dhbt-OH and the ninhydrin test.

The results of the measurements demonstrate that the coupling times in this otherwise uncomplicated synthesis proceeds effectively in combination with the chosen presequence.

- 5 The invention described and claimed herein is not to be limited in scope by the specific embodiments herein disclosed, since these embodiments are intended as illustrations of several aspects of the invention. Any equivalent embodiments are intended to be within the scope of this invention. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from
10 the foregoing description. Such modifications are also intended to fall within the scope of the appended claims.

Various references are cited herein, the disclosure of which are incorporated by reference in their entireties.

15

WHAT IS CLAIMED IS:

1. A process for the production of a peptide having the structure



wherein AA is an L- or D-amino acid residue,

X is hydrogen or an amino protective group

10 Y is OH, NH₂ or an amino acid sequence comprising from 3 to 9 amino acid residues and n is an integer greater than 2 by solid phase synthesis comprising

(a) coupling the C-terminal amino acid in the form of an N-alpha-protected reactive derivative to the support optionally by means of a linker, wherein said C-terminal amino acid comprises a presequence comprising from 3 to 9 amino acids independently selected from native L-amino acids having a side chain functionality which is protected during the coupling steps and having a propensity factor $P\alpha > 0.57$ and a propensity factor $P\beta \leq 1.10$ or the corresponding D-amino acids;

20 (b) adding subsequent amino acids forming the peptide sequence by stepwise coupling or coupling as a peptide fragment in the form of protected fragments;

(c) removing the protecting groups from the peptide sequence of (b) and

25 (d) optionally cleaving the presequence from the formed peptide.

2. The process according to claim 1, wherein the presequences have from 5 to 7 amino acid residues.

3. The process according to claim 1, wherein the C-terminal amino acid is side-chain protected.

5 4. The process according to claim 1, wherein the amino acids forming part of the presequence are independently selected from the group consisting of Lys, Glu, Asp, Ser, His, Asn, Arg, Met and Gln.

10 5. The process according to claim 1, wherein the amino acids in the presequence are either exclusively Lys or Glu or a sequence (Glu)_q(Lys)_p, where p + q is 3 to 9, and the order of Lys and Glu is arbitrarily chosen.

15 6. The process according to claim 1, wherein the amino acids in the presequence are a sequence (Glu)_q(Lys)_p, where p + q is 6 to 9, and the order of Lys and Glu is arbitrarily chosen.

7. The process according to claim 1, wherein the N- α amino protective group is Fmoc or Boc.

20 8. The process according to claim 1, wherein the amino acids in the presequence are chosen from amino acids having a side chain functionality selected from the group consisting of a carboxy, carboxamido, amino, hydroxy, guanidino, sulphide and imidazole moiety.

25 9. The process according to claim 1, wherein the solid support is a functionalized resin selected from the group consisting of polystyrene, polyacrylamide, polyethyleneglycol, cellulose, polyethylene, latex and dynabeads.

10. The process according to claim 1, wherein the C-terminal amino acid is attached to the solid support by means of a common linker selected from the group consisting of 2,4-dimethoxy-4-hydroxy-benzophenone, 4-(4-hydroxymethyl-3-methoxyphenoxy)-butyric acid (HMPB), 4-hydroxymethylbenzoic acid, 4-hydroxymethyl-3-phenoxyacetic acid (HMPA), 3-(4-hydroxymethylphenoxy)propionic acid and p-[(R,S)- α -(9H-fluoren-9-yl)-methoxyformamido]-2,4-dimethoxybenzyl)-phenoxyacetic acid.

11. The process according to claim 1, wherein the peptide is cleaved from the support by means of an acid, a base or by means of photolysis.

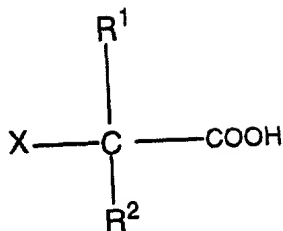
12. The process according to claim 1, wherein the peptide is cleaved from the support by means of an acid selected from the group consisting of trifluoroacetic acid (TFA), trifluoromethanesulfonic acid (TFMSA), hydrogen bromide (HBr), hydrogen chloride (HCl) and hydrogen fluoride (HF).

13. The process according to claim 1, wherein the peptide is cleaved from the support by means of a base selected from the group consisting of ammonia, hydrazine, an alkoxide and hydroxide.

14. The process according to claim 1, in which a linker is inserted between the presequence attached to a support and the AA₁-AA_n sequence.

15. The process according to claim 14, wherein the linker is optically active.

16. The process according to claim 14, wherein the linker is an α -hydroxy or α -amino acid of the general formula



5

wherein X is OH or NH₂, and R¹ and R² are independently selected from H, C₁₋₃ alkyl, phenyl and substituted phenyl, where the substituents are one or more electron donating substituents chosen among C₁₋₃ alkoxy, C₁₋₃ alkyl, or two vicinal substituent groups are joined to form a 5 or 6 membered carbon ring together with the carbon atoms to which they are attached.

10

17. The process according to claim 14, wherein the linker is (+)-4-methoxymandelic acid, diphenylglycine or glycolic acid.

15

18. The process according to claim 1, wherein the presequence is enzymatically cleaved from the formed peptide.

19. The process according to claim 18, wherein the enzyme is selected from the group consisting of suitable carboxy- and endopeptidases.

20

20. The process according to claim 1, further comprising inserting a first linker between the presequence attached to a support and the AA₁-AA_n sequence and a second linker between the presequence and the solid support with orthogonal cleavage conditions to the first linker so the second linker is selectively cleaved by means of an acid or base to give a peptide AA₁-AA_n linked to the presequence by means of said first linker.

25

21. An agent for use in solid phase peptide synthesis having the general formula X-AA'₁...-AA'_m-Y₁-R, wherein R is a solid support applicable in solid phase peptide

synthesis, Y_1 is an amino acid sequence comprising from 3 to 9, independently selected from L-amino acids having a side chain functionality which is protected during the coupling steps and having a propensity factor $P\alpha > 0.57$ and a propensity factor $P\beta \leq 1.10$, or the corresponding D-amino acid, AA' is an L or D-amino acid residue, m is zero or an integer from 1 to 40 and X is hydrogen or an amino protective group.

22. An agent for use in solid phase peptide synthesis having the general formula $X-AA'_1-\dots-AA'_m-L_1-Y_1-R$

wherein R is a solid support applicable in solid phase peptide synthesis, Y_1 is an amino acid sequence comprising from 3 to 9, independently selected from L-amino acids having a side chain functionality which is protected during the coupling steps and having a propensity factor $P\alpha > 0.57$ and a propensity factor $P\beta \leq 1.10$, or the corresponding D-amino acid, AA' is an L or D-amino acid residue, m is zero or an integer from 1 to 40 and X is hydrogen or an amino protective group and L_1 is a linker which enables a selective cleavage of the bond to AA'_m .

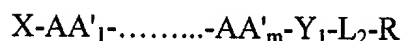
23. An agent for use in solid phase peptide synthesis having the general formula

$X-AA'_1-----AA'_m-L_1-Y_1-L_2-R$

wherein R is a solid support applicable in solid phase peptide synthesis, Y_1 is an amino acid sequence comprising from 3 to 9 amino acid residues independently selected from L-amino acids having a side chain functionality which is protected during the coupling steps and having a propensity factor $P\alpha > 0.57$ and a propensity factor $P\beta \leq 1.10$, or the corresponding D-amino acid, AA' is an L or D-amino acid residue, m is zero or an integer from 1 to 40 and X is hydrogen or an amino protective group, L_1 is a linker which enables a selective cleavage of the bond to AA'_m and L_2 is a linker with orthogonal cleavage

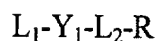
conditions relative to the first linker so that it is selectively cleaved from the solid support.

24. An agent for use in solid phase peptide synthesis having the general formula



wherein R is a solid support applicable in solid phase peptide synthesis, Y_1 is an amino acid sequence comprising from 3 to 9 amino acid residues independently selected from L-amino acids having a side chain functionality which is protected during the coupling steps and having a propensity factor $P\alpha > 0.57$ and a propensity factor $P\beta \leq 1.10$, or the corresponding D-amino acid, AA' is an L or D-amino acid residue, m is zero or an integer from 1 to 40 and X is hydrogen or an amino protective group, L_2 is a linker with orthogonal cleavage conditions relative to the first linker and enabling a selective cleavage from the solid support.

25. An agent for use in solid phase peptide synthesis having the formula



Wherein R is a solid support applicable in solid phase peptide synthesis, Y_1 is an amino acid sequence comprising from 3 to 9 amino acids independently selected from L-amino acids having a side chain functionality which is protected during the coupling steps and having a propensity factor $P\alpha > 0.57$ and a propensity factor $P\beta \leq 1.10$, or the corresponding D-amino acid, L_1 is a linker which enables a selective cleavage of the bond to a peptide sequence and L_2 is a linker with orthogonal cleavage conditions relative to the first linker and enabling a selective cleavage from the solid support.

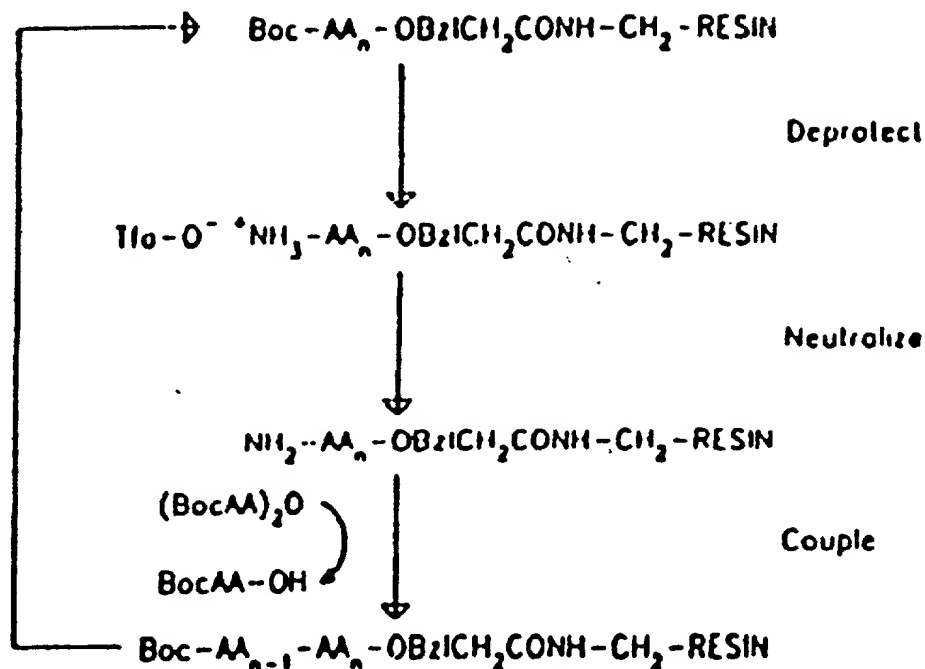
Figure 6

5 solid-phase peptide synthesis.

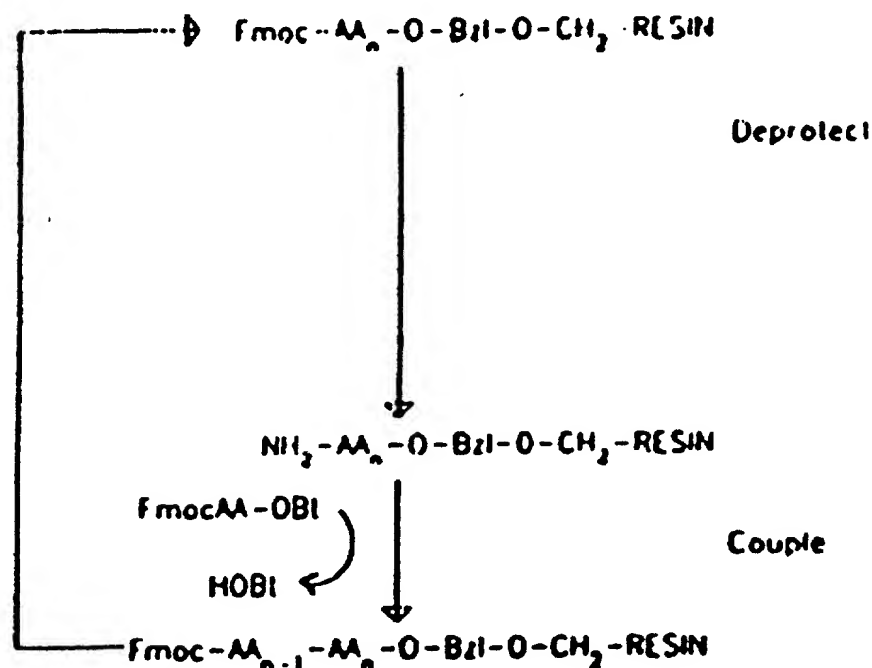
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Figure 1

A.



B.



008140-9225560

Figure 3

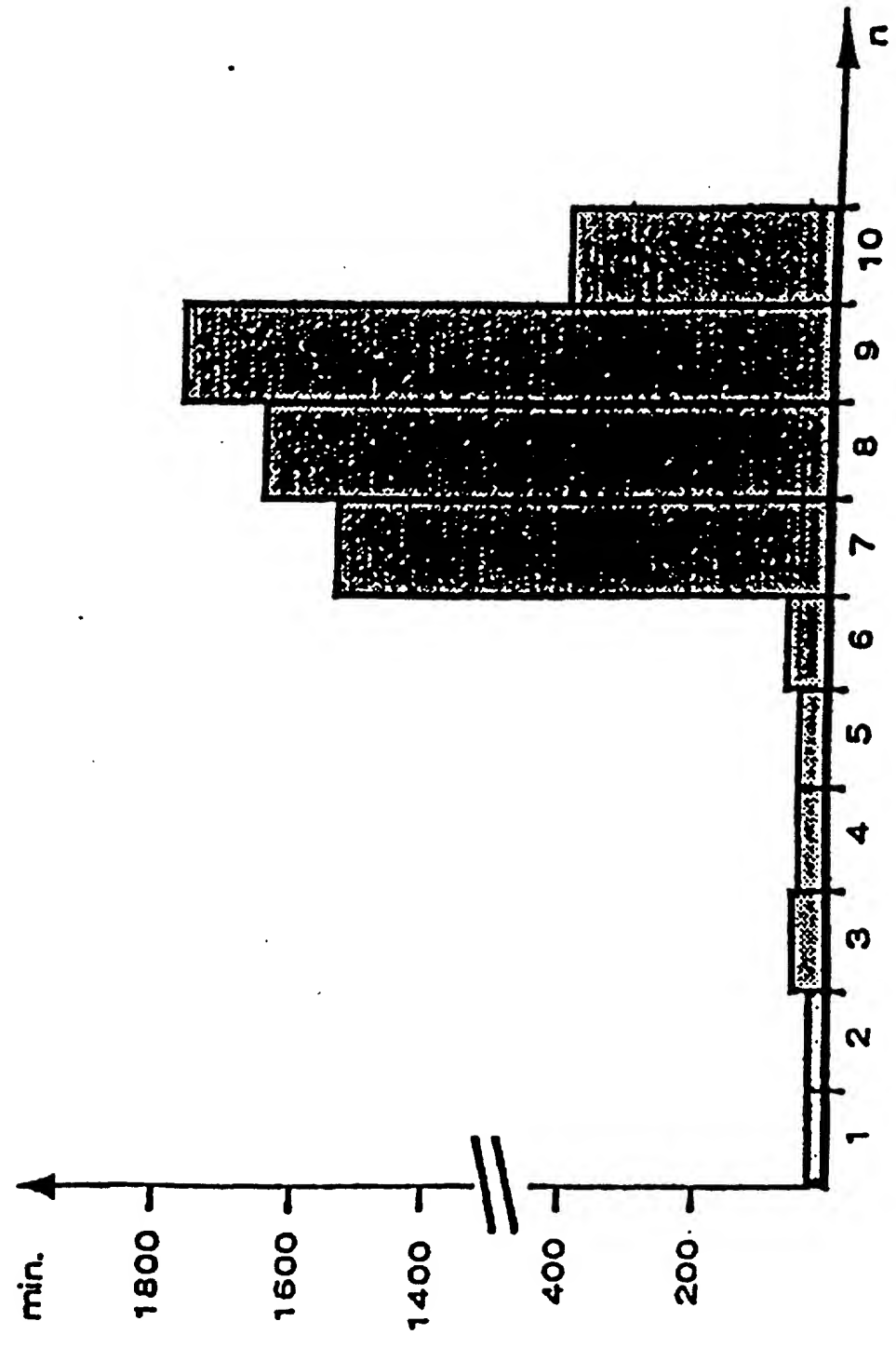
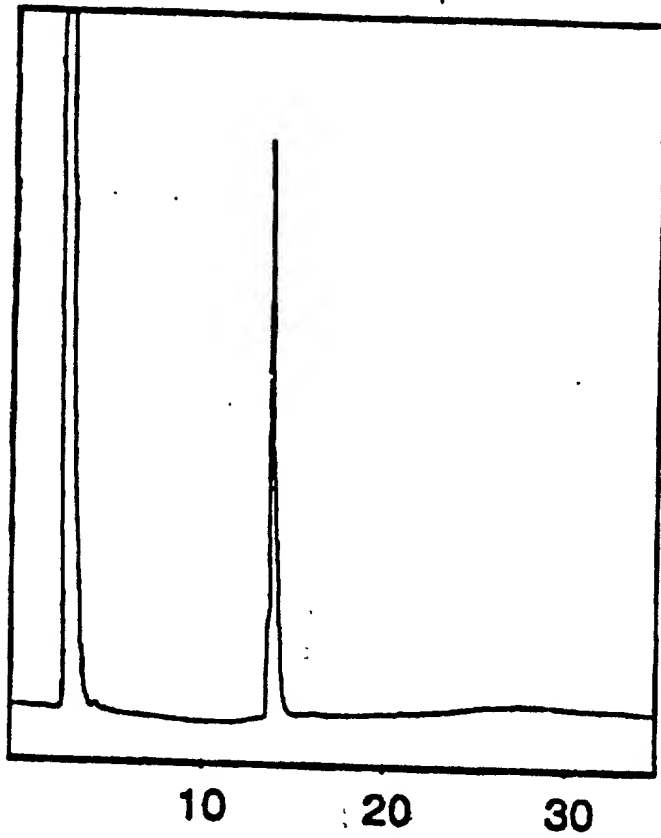
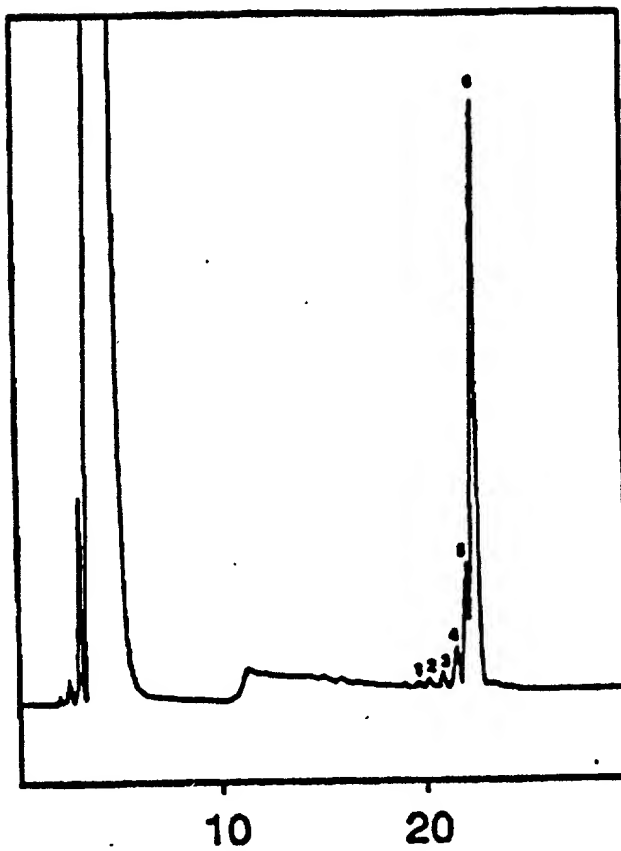


Figure 4



Sheet 5 of 12

Figure 5

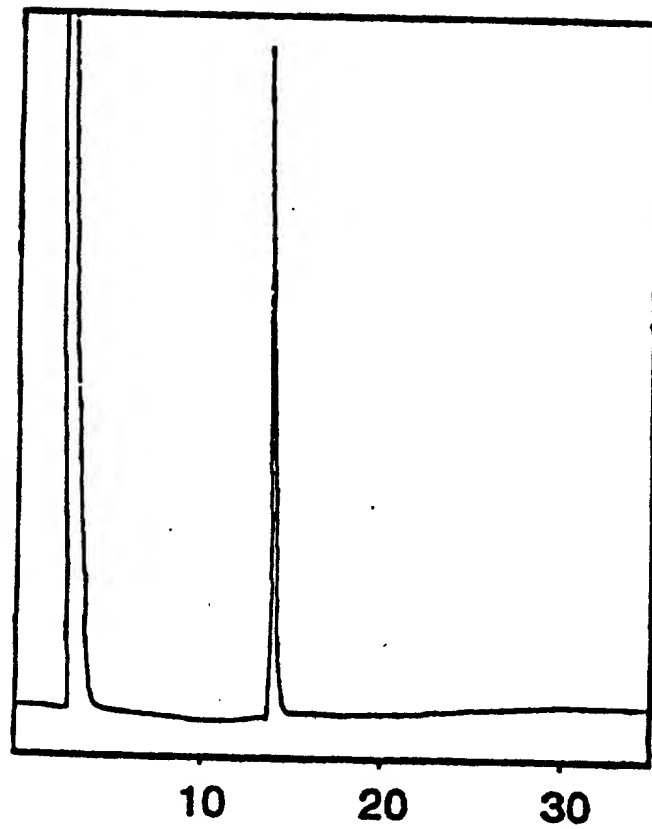


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Sheet 6 of 12

Figure 6



003740" sheet 560

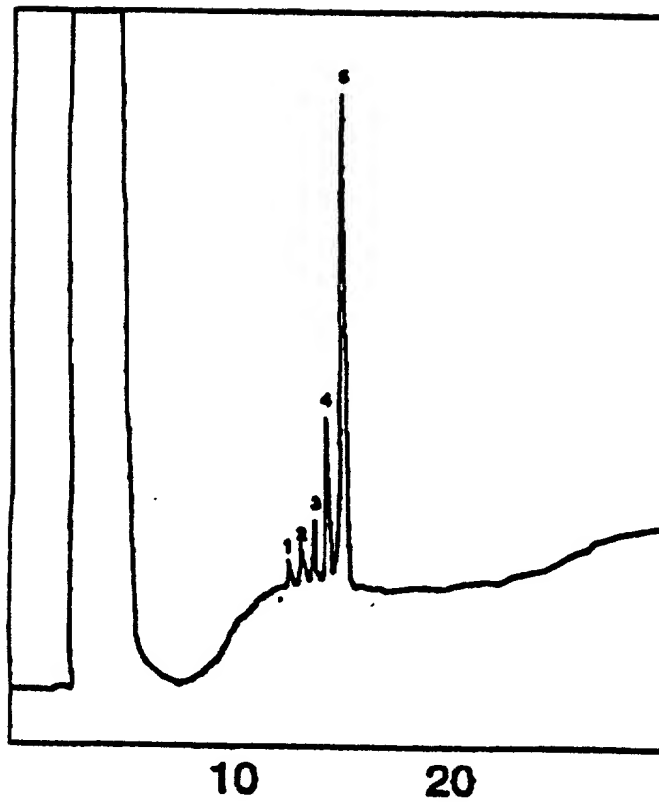
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Figure 7

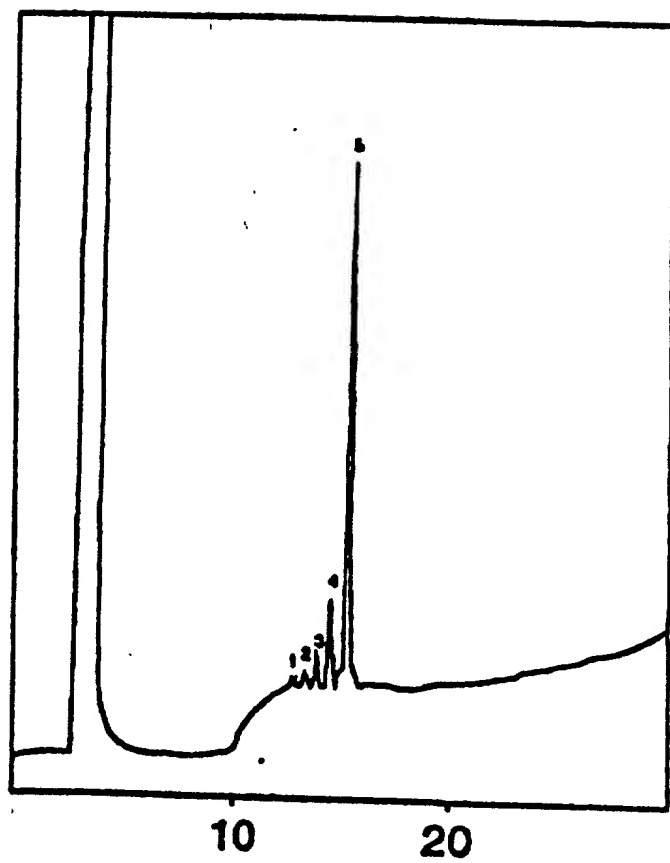


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Figure 8

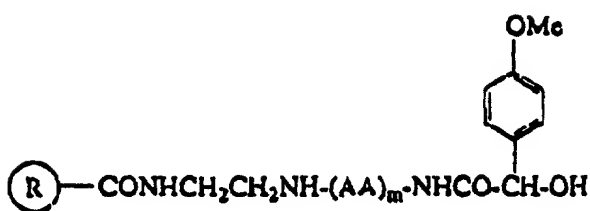
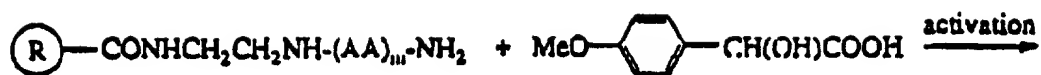


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Sheet 10 of 12

Figure 10



1. Fmoc-deprotection
2. Coupling with 1st Fmoc-amino acid etc.

Figure 11

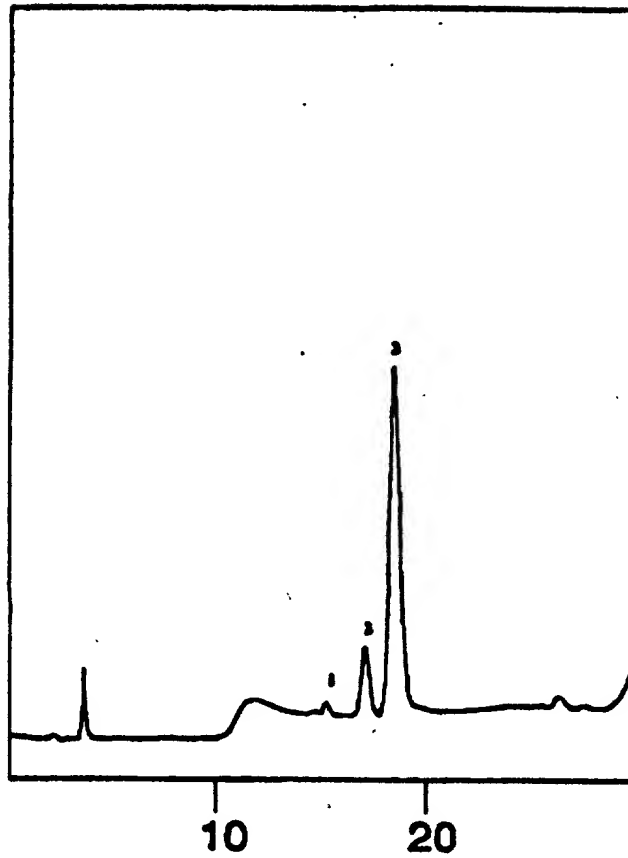
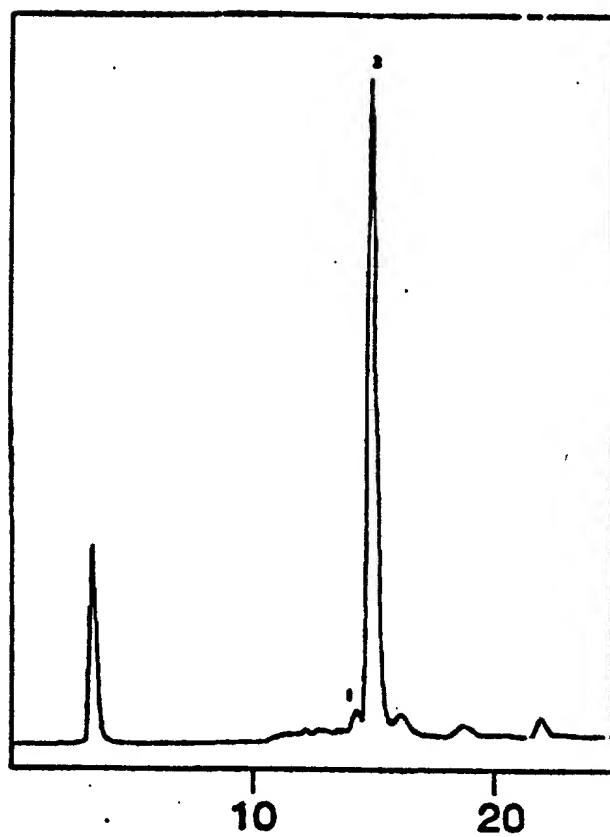


Figure 12



COMBINED DECLARATION FOR PATENT APPLICATION AND POWER OF ATTORNEY
(Includes Reference to PCT International Applications)

Attorney's Docket Number

PPT-1996000050-US-CIP-1

As a below named inventor, I/We hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

Improved Solid-Phase Peptide Synthesis and Agent for Use in Such Synthesis

the specification of which (check only one item below):

☒ is attached hereto

☐ was filed as United States application

Application No. _____

on _____

and was amended

on

☐ was filed as PCT international application

Number _____

on _____

and was amended under PCT Article 19

on

I/We hereby state that I/We have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I/We acknowledge the duty to disclose information which is material to patentability of this application in accordance with Title 37, Code of Federal Regulations, §1.56.

I/We hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate or of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application(s) for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) of which priority is claimed:

PRIOR FOREIGN/PCT APPLICATION(S) AND ANY PRIORITY CLAIMS UNDER 35 U.S.C. 119:

COUNTRY (if PCT, indicate "PCT")	APPLICATION NUMBER	DATE OF FILING (day, month, year)	PRIORITY CLAIMED UNDER 35 USC 119
Denmark	0971/96	9 September 1996	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO
			<input type="checkbox"/> YES <input type="checkbox"/> NO
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COMBINED DECLARATION FOR PATENT APPLICATION AND POWER OF ATTORNEY (Includes Reference to PCT International Applications)				Attorney's Docket Number PPT-1996000050-US-CIP-1	
I hereby claim the benefit under Title 35, United States Code 120 of any United States application(s) or PCT international application(s) designating the United States of America that is/are listed below and, insofar as the subject matter of each of the claims of this applications is not disclosed in that/those prior application(s) in the manner provided by the first paragraph of Title 35, United States Code, 112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, 1.56(a) which occurred between the filing date of the prior application(s) and the national or PCT international filing date of this application:					
PRIOR U.S. APPLICATIONS OR PCT INTERNATIONAL APPLICATIONS DESIGNATING THE U.S. FOR BENEFIT UNDER 35 U.S.C. 120:					
U.S. APPLICATIONS				STATUS (Check one)	
U.S. APPLICATION NUMBER	U.S. FILING DATE		Patented	Pending	Abandoned
09/254,523	March 8, 1999			x	
PCT APPLICATIONS DESIGNATING THE U.S.					
APPLICATION NO.	FILING DATE	US SERIAL NUMBERS ASSIGNED (if any)			
PCT/DK97/00375	9 September 1997	09/254,523			x
POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith. Cheryl H. Agris Reg. No. 30,335					
Send Correspondence to: Cheryl H. Agris, Ph.D. Attorney at Law P.O. Box 806 Pelham, N.Y. 10803				Direct Telephone Calls To: Cheryl H. Agris (914) 712-0093	
1	Full Name of Inventor	Family Name Holm	First Given Name Arne	Second Given Name	
	Residence & Citizenship	City Skodsborg	State or Foreign Country Denmark	Country of Citizenship Denmark	
	Post Office Address	Post Office Address Skodsborgparken 20 II tv	City Skodsborg	State & Zip Code/Country DK-2942 Denmark	
2	Full Name of Inventor	Family Name Larsen	First Given Name Bjarne	Second Given Name Due	
	Residence & Citizenship	City Brønshøj	State or Foreign Country Denmark	Country of Citizenship Denmark	
	Post Office Address	Post Office Address Arildsgård 5, 1.th.	City Brønshøj	State & Zip Code/Country DK-2700 Denmark	

COMBINED DECLARATION FOR PATENT APPLICATION AND POWER OF ATTORNEY (Includes Reference to PCT International Applications)				Attorney's Docket Number PPT-1996000050-US-CIP-1	
3	Full Name of Inventor	Family Name	First Given Name	Second Given Name	
	Residence & Citizenship	City	State or Foreign Country	Country of Citizenship	
	Post Office Address	Post Office Address	City	State & Zip Code/Country	
4	Full Name of Inventor	Family Name	First Given Name	Second Given Name	
	Residence & Citizenship	City	State or Foreign Country	Country of Citizenship	
	Post Office Address	Post Office Address	City	State & Zip Code/Country	
5	Full Name of Inventor	Family Name	First Given Name	Second Given Name	
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6	Full Name of Inventor	Family Name	First Given Name	Second Given Name	
	Residence & Citizenship	City	State or Foreign Country	Country of Citizenship	
	Post Office Address	Post Office Address	City	State & Zip Code/Country	
7	Full Name of Inventor	Family Name	First Given Name	Second Given Name	
	Residence & Citizenship	City	State or Foreign Country	Country of Citizenship	
	Post Office Address	Post Office Address	City	State & Zip Code/Country	
8	Full Name of Inventor	Family Name	First Given Name	Second Given Name	
	Residence & Citizenship	City	State or Foreign Country	Country of Citizenship	
	Post Office Address	Post Office Address	City	State & Zip Code/Country	
9	Full Name of Inventor	Family Name	First Given Name	Second Given Name	
	Residence & Citizenship	City	State or Foreign Country	Country of Citizenship	
	Post Office Address	Post Office Address	City	State & Zip Code/Country	

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.


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<110> Holm, Arne
      Larsen, Bjarne

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